Bioactivity of *Bacillus* Species Isolated from Human Feces

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Abstract

In the present study, twenty isolates of *Bacillus* genus according to morphological feature of colonies and the presence of spores by light microscopy, were obtained from 15 samples of human feces, after heat-treated and killed all vegetative cells. These spore-former isolates were characterized at the species level biochemically by the use of API 50 CHB kit and identified as *Bacillus insolitus, B. laterosporus, B. polymyxa* and *B. bodius* with proportion ratio rate 40, 20, 35 and 5% of the total, respectively. Eighty five percent of *Bacillus* isolates were formed biofilm, which have protective and adhesive properties and could be responsible for an increased antibiotics resistance. Seventy five percent of *Bacillus* species isolated from human feces were produced inducible extracellular Levansucrase and responsible for synthesis of fructan polymers (levan), described as useful prebiotics due to the capacity of the β-linked fructose units. Five *Bacillus* species were showed positive result for Exopolysaccharides production. *Bacillus* species resistant to Ampicillin (AP), Chloramphenicol (C), Nitrofurantoin (NI), Gentamicin (GM), Carbenicillin (PY), Nalidixic acid (NA), Methenamine Mandelate (MM), and Cotrimoxazole (TS), reached 20, 100, 60, 0.0, 5, 95, 5 and 70%, respectively.

Keywords: *Bacillus* species, Human feces, Classification, Biofilm. Levansucrase, Exopolysaccharides, Antibiotic susceptibility.

الفعاليات الحيوية لأنواع بكتريا *Bacillus* المعزولة من براز الإنسان

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الخلاصة

جمعت في هذه الدراسة عشرين عزلة من جنس *Bacillus* حسب الصفات الشكلية للمستعمرات وتمييز السبورات بواسطة المجهر الضوئي، من 15 عينة براز إنسان بعد قتل الخلايا الخضرية بالمعاملة الحرارية. شخصت العزلات المكونة للسبورات كيميائيا باستخدام العدة API 50 CHB إلى *B. insolitus* ,*Bacillus laterosporus* ,*B. polymyxa* و*B. Bodius* بالنسب المئوية 40 و20 و35 و5 %، على التوالي. وجد أن 85% من عزلات *Bacillus* مكونة للفلم الحيوي الذي يمتلك خاصية الوقاية والالتصاق وقد يكون مسؤولا عن زيادة المقاومة للمضادات الحيوية. كما وجد ان 75% من أنواع *Bacillus* المعزولة من براز الإنسان منتجة لأنزيم Levansucrase المفرز خارج الخلية والمسؤول عن تخليق بوليمرات الفركتوز (الليفان) الموصوفة كمادة غذائية مفيدة للمعززات الحيوية. تبين أن خمسة أنواع من *Bacillus* منتجة للمواد البوليمرية الخارجية إضافة إلى مقاومتها للمضادات الحيوية التالية (AP) Ampicillin، (C) Chloramphenicol، (NI) Nitrofurantoin، (GM) Gentamicin، (PY) Carbenicillin، (NA) Nalidixic acid، (MM) Methenamine Mandelate، (TS) Cotrimoxazole، بالنسب المئوية 20، 100، 60، 0.0، 5، 95، 5 و 70 ، على التوالي.

الكلمات المفتاحية : أنواع بكتريا *Bacillus* ، براز الإنسان، التصنيف، الفلم الحيوي، ليفان سكريز، متعدد السكريات الخارجي، المقاومة للمضادات الحيوية.

Introduction

Bacteria belonging to the genus *Bacillus* species are commonly associated with soil, and as such are isolated almost ubiquitously from soil, water, dust, and air. The dominant bacteria found in the small and large intestines are species of Lactobacilli, Streptococci, Enterobacteria, Bifidobacteria, Bacteroides, Clostridia and *Bacillus* species. For example, in a

study of human faeces, *Bacillus* species were isolated in numbers of between 5 × 10^3 and 5 × 10^6 CFU g^{-1} feces [1].

Biofilm are formed by adhesion of cells to surfaces through an exopolymeric matrix. This matrix is important both in the formation and structure of the biofilm, and also on the protection of the cells since it may prevent the access of antimicrobials and xenobiotics to the cells inside the biofilm and confer protection against environmental stresses such as UV radiation, pH shifts, osmotic shock and desiccation [2, 3]. Bacterial strains that do not produce (exopolymeric substance or extracellular polysaccharide or exopolysaccharide) (EPS) present lower adhesive abilities than slime producing strains. EPS is particularly valuable after the initial phase of adhesion in organisms, conferring protection against phagocytosis, interference with the cellular immune response, reduction of antibiotic potency [4, 5] and may protect the cell against unfavorable environmental conditions like oxygen tension, toxic compounds, temperature or high osmotic pressure, and may contribute to the uptake of metal ions [6].

Fructosyloligosaccharides are described as useful probiotics due to the capacity of the β-linked fructose units to pass the gastrointestinal tract undigested. In the colon, they stimulate selectively the growth of beneficial gut bacteria, like Bifidobacteria or Lactobacilli [7]. In addition, fructan, like levan, are employed in the food and non-food industry as viscosifier, stabilizer, emulsifier, gelling, or water-binding agent. Fructooligosaccharides are produced by fructosyltransferases, like Levansucrase. Levansucrase (EC 2.4.10) synthesize levan composed of β-2, 6 linked fructose residues [8].

The functions of intestinal microbiota may include diverse actions in the gastrointestinal tract including nutritional fermentation, participation in the host's immune defense system against pathogens as a probiotic and production of metabolites or enrich metabolites such as glycans, amino acids, xenobiotics, vitamin K, folate and short-chain fatty acids [9].

One action of probiotics is that they can produce antimicrobial substances as direct antagonists against intestinal pathogens. Probiotics may exert their effective antagonistic activity alone or synergistically. Recent studies have indicated that the antagonistic activities against intestinal pathogens are produced by antimicrobial substances from several probiotics strains [10].

The aim of this study is to screen susceptibility of *Bacillus* species human feces for Biofilm, Levansucrase, Exopolysaccharides production and their resistant to antibiotics.

Materials and Methods

Bacterial strains

Spore-forming *Bacillus* isolates were obtained from laboratory of Bacteriology, Department of Biology, Ministry of Science and Technology, Baghdad, Iraq.

Biofilm assay

To test biofilm production overnight, cultures were used to inoculate liquid MSgg medium (100mM l^{-1}MOPS pH 7.0, 0.5% glycerol, 0.5% glutamic acid, 5mM potassium phosphate pH 7.0, 50µgm l^{-1} tryptophan, 50 mgm l^{-1} phenylalanine, 2 mM l^{-1} $MgCl_2$, 0.7 mM l^{-1} $CaCl_2$, 50µM l^{-1} $FeCl_3$, 50µM l^{-1} $MnCl_2$, 2µM l^{-1} thiamine, 1µM l^{-1} $ZnCl_2$) [11]. Cells grown at 37°C without shaking for up to 48h. Cells forming a solid layer at the liquid-air interface were considered as biofilm producers.

Levansucrase production

Screening on solid medium

Bacillus isolates were activated on Luria-Bertani (LB) broth (10gm Bacto-tryptone, 5gm Bacto-yeast extract, 10gm NaCl, completed to one liter of distilled water and pH was adjusted to 7.0), incubation was done at 37°C for 18h, one tenth micro liter of culture suspension was streaked on sucrose mineral salt agar and incubated at 37°C for 48h.

Enzyme production

The following medium was used as cellular production medium [12] for Levansucrase production and had the following composition (g/L): yeast extract 2.5gm, sucrose 200gm, $MgSO_4$ 0.2gm and K_2HPO_4 5.5gm. The medium was completed by addition one liter of distilled water, after adjusted the pH to 7.8.

Enzyme assay

Cells were harvested by centrifuge at 5000 rpm for 10min at 4°C and the supernatant was used as enzyme source. Sucrase activity was assayed by analyzing the reducing sugar liberated during sucrose hydrolysis. The reaction mixture [250µl of enzyme extract and 250µl of 1M sucrose in acetate buffer (50mM, pH 5.0)] was incubated at 30°C for 30min. Reducing sugar

released was determined according to Somogyi [13].

One unit of Levansucrase activity was expressed as the amount of enzyme required to liberate 1µmol of reducing sugar from sucrose in 1min under experimental conditions.

Production of Exopolysaccharides

The cultures were streaked on Yeast Malt Glucose agar (YMG) agar plates (Composition per liter: 10.0gm glucose, 3.0gm yeast extract, 3.0gm malt extract, 5.0gm peptone, agar 15gm, and pH was adjusted to 7.0). After incubating at 30°C for 48h the plates were examined for the presence of mucous colonies [14].

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by the disc diffusion method [15]. The test was performed on Muller Hinton agar using antibiotic impregnated discs. The following eight antibiotic discs were used in the test: Ampicillin (AP), Chloramphenicol (C), Nitrofurantoin (NI), Gentamicin (GM), Carbenicillin (PY), Nalidixic acid (NA), Methenamine Mandelate (MM), Cotrimoxazole (TS). After 18 hours of incubation at 37°C, the strains were characterized as susceptible or resistant based on the zone of inhibition created around the discs.

Results and Discussion

Identification of spore-former Bacilli from human feces

Twenty isolates of *Bacillus* genus according to morphological feature of colonies and the presence of spores by light microscopy, used in this study (Table 1) were *B. insolitus, B. laterosporus, B. polymyxa and B. bodius* with (40, 20, 35 and 5)% of the total, respectively.

Biofilm production

Results showed that 85% of strains belong to *B. insolitus, B. polymyxa* and *B. laterosporus* with (17 out of 20) formed biofilm (Fig. 1). This is an interesting observation since biofilm have protective and adhesive properties and have been associated with a longer persistence of Bacilli in the gastrointestinal tract (GIT) of animals, then prevent the access of antimicrobials and xenobiotics to the cells inside the biofilm and confer protection against environmental stresses such as UV radiation, pH shifts, osmotic shock and desiccation [17]. In fact, the host immune system is, in general, capable of rapidly kill non-adherent bacteria. The slow growth rate observed in biofilm and/or transport limitations of nutrients, metabolites and oxygen between the

surface and the interior of the biofilm could be responsible for an increased antibiotic resistance over planktonic cells [18].

Fructan polymers production

In this study 75% of *Bacillus* species isolated from human feces were secreted inducible extracellular Levansucrase, appeared as mucoid consistence of bacterial colonies on sucrose mineral salt agar by using sucrose as carbon source (Table 2). A large amount of levan was produced when the bacteria were cultivated using sucrose, but the yields varied with the sucrose concentration. Enzymes responsible for the synthesis of fructan polymers of the levan type are generally referred to as fructosyltransferases (FTF) or Levansucrase. They catalyse the transfer of the fructosyl unit of sucrose to a number of acceptors including sucrose, water (resulting in hydrolysis) and fructan polymer [19]. So fructosyloligosaccharides are described as useful prebiotics due to the capacity of the β-linked fructose units to pass the gastrointestinal tract undigested. In the colon, they stimulate selectively the growth of beneficial gut bacteria, like *Bifidobacteria* or Lactobacilli [7].

Exopolysaccharides production

Out of 20 isolates only five were showed positive result for Exopolysaccharides on YMG agar (Fig. 2). Microorganisms synthesize large spectrum multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides (EPSs). Exopolysaccharides generally consist of monosaccharide and some non carbohydrate substituent's (such as protein, nucleic acids, lipids, acetate, pyruvate, succinate, and phosphate). Microbial EPS plays an important task in interaction between bacteria and their environment [20]. EPS showed higher stability against enzymatic degradation, capability for metal removing, and heat stable. It may find possible applications in the industrial fields and in biotechnological processes [21].

Antibiotic resistance

The antibiotic resistance pattern showed that *Bacillus* species isolated from human feces were resist to Ampicillin (AP), Chloramphenicol (C), Nitrofurantoin (NI), Gentamicin (GM), Carbenicillin (PY), Nalidixic acid (NA), Methenamine Mandelate (MM), Cotrimoxazole (TS), with proportion ratio rate (20, 100, 60, 0.0, 5, 95, 5 and

70)%, respectively (Table 2). *Bacillus* specie showed multiple drug resistance (MAR) against four tested antibiotics (More than 50%) appeared with Chloramphenicol (C), Nitrofurantoin (NI), Nalidixic acid (NA) and Cotrimoxazole (TS). Regardless of whether the resistance is mediated by excessive or multiple exposures to antibiotics by other mechanisms, the antibiotic resistant bacteria may have public health significance. The selective process leading to the emergence and maintenance of bacterial resistance to antibiotics are mainly brought about by incorrect or abusive utilization of the drugs [22].

Table 1. Strains of *Bacillus* spp. isolated from feces [16].

Strains	Species
AS1	*Bacillus insolitus*
AS2	*Bacillus laterosporus*
AS3	*Bacillus insolitus*
AS4	*Bacillus laterosporus*
AS5	*Bacillus laterosporus*
AS6	*Bacillus polymyxa*
AS7	*Bacillus laterosporus*
AS8	*Bacillus insolitus*
AS9	*Bacillus insolitus*
AS10	*Bacillus polymyxa*
AS11	*Bacillus insolitus*
AS12	*Bacillus polymyxa*
AS13	*Bacillus polymyxa*
AS14	*Bacillus polymyxa*
AS15	*Bacillus bodius*
AS16	*Bacillus polymyxa*
AS17	*Bacillus insolitus*
AS18	*Bacillus insolitus*
AS19	*Bacillus polymyxa*
AS20	*Bacillus insolitus*

*Species assignment was based on the results of the API 50 CHB kit.

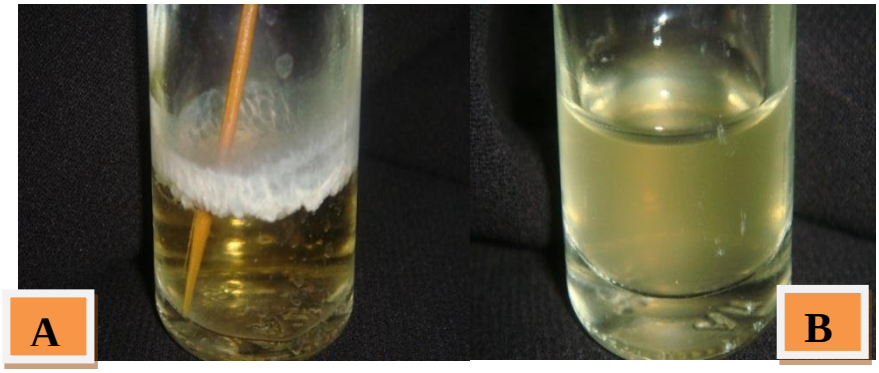

Figure (1) Biofilm production, (A) positive, (B) negative

Table 2. *Bacillus* species levansucrase production

Strains	Species	Levansucrase production	Levansucrase activity Unit / ml
AS1	*Bacillus insolitus*	+	92.096
AS2	*Bacillus laterosporus*	-	-
AS3	*Bacillus insolitus*	+	92.153
AS4	*Bacillus laterosporus*	-	-
AS5	*Bacillus laterosporus*	-	-
AS6	*Bacillus polymyxa*	+	90.893
AS7	*Bacillus laterosporus*	-	-
AS8	*Bacillus insolitus*	+	93.264
AS9	*Bacillus insolitus*	+	88.453
AS10	*Bacillus polymyxa*	+	89.553
AS11	*Bacillus insolitus*	+	92.886
AS12	*Bacillus polymyxa*	+	97.216
AS13	*Bacillus polymyxa*	+	95.945
AS14	*Bacillus polymyxa*	+	91.855
AS15	*Bacillus bodius*	-	-
AS16	*Bacillus polymyxa*	+	92.577
AS17	*Bacillus insolitus*	+	94.158
AS18	*Bacillus insolitus*	+	93.092
AS19	*Bacillus polymyxa*	+	92.096
AS20	*Bacillus insolitus*	+	94.432

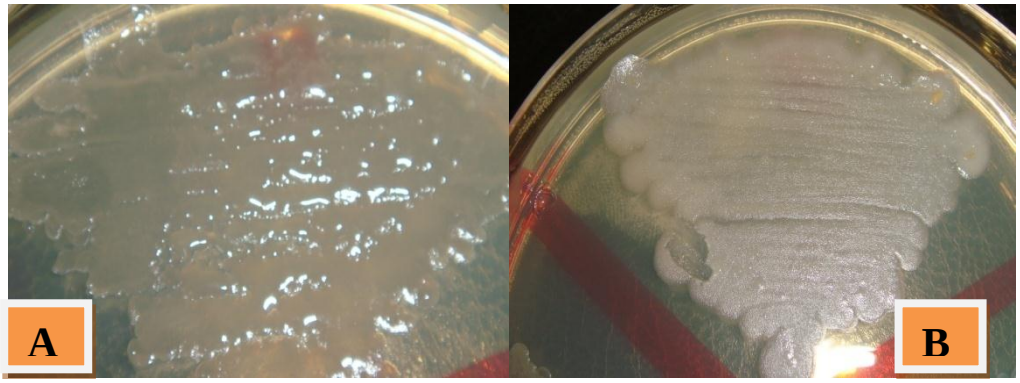

Figure 2. Exopolysaccharides production
(A) positive, (B) negative

Table 3. Antibiotic susceptibility testing for human feces *Bacillus* species

Strains	Species	Antibiotics (mm)							
		AP	C	NI	GM	PY	NA	MM	TS
AS1	*Bacillus insolitus*	10	18	10	S	S	30	S	30
AS2	*Bacillus laterosporus*	S	20	17	S	S	20	S	27
AS3	*Bacillus insolitus*	S	15	18	S	S	15	S	25
AS4	*Bacillus laterosporus*	S	18	8	S	S	13	S	S
AS5	*Bacillus laterosporus*	S	13	15	S	S	22	S	30
AS6	*Bacillus polymyxa*	12	30	S	S	S	20	S	25
AS7	*Bacillus laterosporus*	15	28	S	S	S	15	S	20
AS8	*Bacillus insolitus*	10	28	S	S	10	20	S	25
AS9	*Bacillus insolitus*	S	17	S	S	S	25	S	27
AS10	*Bacillus polymyxa*	S	15	13	S	S	17	S	20
AS11	*Bacillus insolitus*	S	15	10	S	S	10	S	17
AS12	*Bacillus polymyxa*	S	17	S	S	S	26	S	26
AS13	*Bacillus polymyxa*	S	17	S	S	S	17	S	S

AS14	*Bacillus polymyxa*	S	15	S	S	S	23	S	S
AS15	*Bacillus bodius*	S	19	10	S	S	S	S	15
AS16	*Bacillus polymyxa*	S	17	S	S	S	17	S	S
AS17	*Bacillus insolitus*	S	25	12	S	S	17	S	10
AS18	*Bacillus insolitus*	S	19	10	S	S	19	S	17
AS19	*Bacillus polymyxa*	S	17	12	S	S	16	S	S
AS20	*Bacillus insolitus*	S	25	13	S	S	20	8	S
Proportion ratio rate of antibiotic resistance %		20	100	60	0.0	5	95	5	70

R: Resistance, S: Sensitive, AP : Ampicillin, C: Chloramphenicol,
NI : Nitrofurantoin, GM : Gentamicin, PY: Carbenicillin,
NA: Nalidixic acid, MM: Methenamine Mandelate, TS: Cotrimoxazole

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