JJBS

Volume 6, Number 4, December .2013
ISSN 1995-6673
Pages 272 - 276

Jordan Journal of Biological Sciences

Correlation Between Numerical Profiles Generated for Soil Spore Forming Bacilli and Their Inhibitory Potential Against *Staphylococcus aureus* ATCC 6538

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Received: May 9, 2013 Revised: June 18, 2013 Accepted June 21, 2013

Abstract

Numerical profiling using conventional tests for studying bacterial diversity of soil have probably never been employed. The main objective of this work was to describe the heterogeneity of spore forming bacilli in a vegetative soil using numerical profiling and to establish the possible application of this approach for the prediction of the antimicrobial potentials of soil dwelling *Bacillus* species. A total of 100 spore forming Gram positive bacilli were recovered from soil samples and their characters were determined using 9 conventional tests. A 3 digit numerical profile was constructed for each isolate. This labelling system resulted in the generation of 12 different profiles which indicated the diversity of soil *Bacillus* isolates. The highest frequency of occurrence was detected among the isolates labelled with the profile 4.1.5 and the lowest among those labelled with 4.7.5; 4.6.6 and 1.0.3 profiles. Out of 13 isolates labelled with 4.4.3, the cell free extract of 12 exhibited no antimicrobial activity against *Staphylococcus aureus* ATCC 6538 whereas, extracts obtained from 15 out of 18 isolates labelled with the profile 4.1.5 were inhibitory to the test organism. It is concluded that the numerical profiling system is a significant tool for the establishment of diversity among soil spore forming bacilli which can also be used for the prediction of the potentials of isolates to produce antimicrobial metabolites.

Keywords: *Bacillus* species, Vegetative soil, Diversity, Antimicrobial effect, Numerical profiles.

1. Introduction

Bacillus species are soil dwelling organisms that produce metabolites with diverse chemical structures and potentials to inhibit a wide spectrum of bacteria (Morsi and Atef, 2010). Antibiotics produced by Bacillus species include bacitracin, colistin, circulin, gramycidin, polymyxin, and tyrotricidin. However, these antibiotics were discovered many decades ago, they are still used in medical practice (Fickers, 2012).

The isolation of *Bacillus* species from soil is usually carried out by traditional techniques which involve the treatment of soil with heat to inhibit vegetative microorganisms and to allow the selection of spore forming bacteria (Kuta *et al*, 2009). Isolates may be characterized by a range of methods. Morphological and physiological characteristics were used for classification and identification. However, such techniques can provide the basis for bacterial, and *Bacillus* taxonomy, additional tests were found to be necessary for confirmation and for fine-scale resolution (Maughan and Van der Auwera, 2011).

Identification of spore forming bacteria is a difficult task, it was suggested that for the accurate identification of these Bacilli, several standard schemes should be used together for the establishment of different phenotypic characteristics of all known species (Morsi *et al*, 2010). In recent years, molecular techniques were introduced for the characterization of isolates at a genetic level (Kadyan *et al*, 2013). Although, this characterization approach is valuable in discriminating and grouping *Bacillus* strains, it provides little ecological information. For example, 16S rRNA gene based methods provides good phylogenetic information to the genus level, but in themselves, give little information on function (Mandic-Mulec and Prosser, 2011; Maughan and Van der Auwera, 2011). On the other hand, traditional approaches that group isolates on the basis of common metabolic properties

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may be limited in terms of phylogenetic power but provide clues to environmental factors and can be strong indicators of potential ecosystem function (Koeppel *et al*, 2008). Kim *et al.* (2003) indicated that for the accurate identification and classification of spore forming Bacilli from soil, a multiphasic approach may be required.

Antimicrobial activity is usually established by screening cell free extract derived from soil isolates against a panel of bacteria using the agar diffusion method (Manga and Oyeleke, 2008). Therefore, it is evident that identification procedures in addition to preliminary screening are tedious and time consuming. If a system or a model can be devised to label the isolates and simultaneously predict their potential to produce antimicrobial substances, then definitely; time, efforts and money can be saved. The effective labeling system should be capable of grouping isolates according to their biochemical properties and should detect variations among isolates. These stipulations were taken into considerations by (Griffiths and Lovitt, 1980), who suggested that numerical profiling can be used as a significant tool for the study of bacterial diversity in different ecosystems. The advantage of this system over traditional and modern identifications methods is that all isolates are labeled and none is left unidentified. Depending on the tests included in the characterization of isolates, it can provide an idea about the function of the isolate from the respective environment. The objectives of this communication were to establish the diversity of endospore forming *Bacillus* species recovered from rich vegetative Jordanian soil using the numerical profiling system and to investigate the possible application of this system for the prediction of antimicrobial potential of these isolates.

2. Materials and Methods

2.1. Collection of Soil Samples

A total of 10 soil samples were collected from highly vegetative locations in Ajloon Mountains (near the castle). Samples were derived from the depth of 5 cm below the surface, placed in sterile containers and processed in the laboratory within 2 days of collection.

2.2. Isolation of Bacillus Species

One gram of each soil sample was separately suspended in a sterile tube containing 9 ml of sterile distilled water and shaken vigorously for 2 minutes using a vortex mixer. The samples were heat treated at 80 ºC for 10 minutes in a water bath. Upon cooling to room temperature, each soil suspension was 10 fold serially diluted in sterile distilled water. Aliquots of 0.1 ml of the appropriate dilution were plated on Nutrient Agar Medium (Difco- USA) using the spread plate technique. All plates were then incubated at 37 °C for 2 days. From plates that contained counts between 30 to 250 colonies, 10 colonies with apparently different colonial morphology were isolated and further purified by repeated subculture.

2.3. Preparation of Cell Free Extracts

Each purified isolate was inoculated into a sterile tube containing 10 ml of Nutrient Broth (Difco- USA) and then incubated at 30 °C for 72 hours. The tubes were centrifuged and supernatant was then filtered through 0.22 bacteriological filters and stored at 4°C until used as described in the next experiment.

2.4. Antimicrobial Activity Test

Cell suspension of *Staphylococcus aureus* ATCC 6538 was prepared in accordance with the method described by Abu Shaqra and Al Groom (2012) using 0.5 McFarland standard. The suspension prepared, contained 10^6 Colony Forming Unit /ml and was employed to streak plates of Mueller Hinton Agar (Difco- USA). After drying, wells were bored using sterilized cork borer of 6 mm diameter and each well received 50 µl of the extract of a single isolate. Plates were incubated for 24-48 h at 37 ºC before measuring the diameter of inhibition zones developed.

2.5. Numerical Profiles

Tests used to characterize each soil isolate are presented in table 1. These tests were performed as described by Baron *et al* (1994). Results were arranged in groups of 3 and scores of 1, 2, or 4 were assigned to label the isolates according to their position in the table. In this context, attention should be drawn to two important points; first, all tests carried equal weights as they were randomly positioned in the table and second, scoring numbers were chosen to avoid overlap between tests in case of positive results when the total score is calculated. For example, if the first digit was 3, this would indicate that the organism was xylose fermentor, grew in 10% concentration of NaCl and was unable to grow under anaerobic conditions. If the score of the third test in the profile was 3, then one would be confused wither the organism was positive for the first two tests or merely positive for the third test.

The percentage diversity of the *Bacillus* isolates in relation to the profiles generated was calculated according to the equation given by Abu Shaqra and Mashni (2006) as follows:

$$\frac{\text{Number of profiles}}{\text{Total number of isolates}} \times 100$$

The work was concluded by relating the antimicrobial activity of each isolate with its generated numerical profile to determine if the profiles constructed can provide indications regarding the inhibitory activity of the cell free extract derived from the isolates.

Table 1. Characters used to generate numerical profiles for spore forming bacilli recovered from soil samples.

Score	First digit	Second digit	Third digit
0 or 1	Xylose fermentation	Mannose fermentation	Nitrate reduction
0 or 2	Growth in 10% NaCl	Raffinose fermentation	Hipurate hydrolysis
0 or 4	Anaerobic growth	Voges Proskauer	Starch hydrolysis

2.6. Tabulation of Results

Each numerical profile generated has taken a separate row in a table and the numbers of isolates labelled with the respective profile were tabulated next to it. Upon measurement of the antibacterial effect against *Staphylococcus aureus* ATCC 6538, zones of inhibitions were categorized into 3 groups; no zone of inhibition, ≤ 10 mm and > 11 mm. Isolates that belonged to a specific profile generated were grouped according to the size of inhibitory zone determined and were also placed in the table next to their profiles (Table 2). Therefore, it was possible to correlate the number of isolates in each profile with their antimicrobial activity and this is discussed in more details in the results section.

2.7. Statistical Analysis

The system of Microsiris statistical analysis and data management was employed. Student t test was performed to establish significance of difference between the number of isolates grouped in each numerical profile and the zone of inhibition measured for the cell free extract derived from each isolate against *S. aureus* ATCC 6538. The test was performed to profiles that contained equal or more than 7 isolates. The difference was considered as significant when Probability (p) value was less than 0.05.

3. Results

A total of 100 pure isolates of bacteria were obtained from 10 soil samples collected from mountainous and highly vegetative region in Jordan. All recovered organisms were spore forming, Gram positive bacilli in pairs or longer chains. Most of the isolated colonies were white, opaque or translucent and they were rough, granular, or wispy. These characteristics were considered as presumptive for the identification of the isolates as *Bacillus* species.

Using the numerical profiling system and the 9 identification tests given in table 1, it was possible to generate 12 different profiles and this indicated the diversity of the *Bacillus* species recovered (12% diversity). Figure 1 illustrates the percentage occurrence of positive characters which constituted the back bone of this diversity. It is evident that 83% of the isolates were capable of nitrate reduction while 67% were starch hydrolysers.

Table 2 demonstrates the frequency of occurrence of each profile among the isolates. This table clearly shows that profile 4.1.5 was the label for the most common isolates whereas; 4.6.6; 4.7.5 and 1.0.3 were the least encountered.

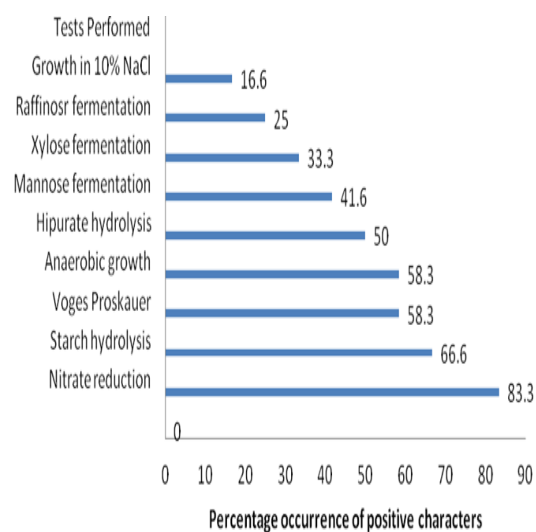

Figure 1. Percentage occurrence of positive characters among spore forming isolates recovered from soil samples.

The diameter of the inhibition zone produced by cell free extract derived from each isolate was taken as a criterion to mark its antimicrobial activity. Table 2 also shows the number of isolates in each profile generated in relation to the zone of inhibition measured. Two examples are given below to explain the content of this table; out of 9 isolates labeled with the profile 2.5.2, 5 produced zones of inhibition > 11 mm, while 3 exhibited zones ≤ 10 mm and only 1 isolate was none inhibitory. On the other hand, 13 isolates were labelled with the profile 4.4.3, 12 of these isolates were inhibitory and none showed the formation of any inhibitory zone.

According to results presented in table 2, 43% of isolates demonstrated no inhibitory zone, while 21% and 36% of the isolates showed inhibitory zone equal or less than 10 and > 11 mm in diameter, respectively. It is worth noting that the majority of *Bacillus* isolates labeled with profiles such as 4.1.5 and 3.4.5 were effective inhibitors to the test organism whereas, the majority of species labelled with 2.1.7, 4.4.3 and 0.0.1 were none inhibitors. Although it was not the intention of this work to identify isolates to species level, it was possible using the few tests performed and the identification tables given by (Barrow and Feltham, 1993) to predict closely the *Bacillus* species to which many of the isolates belonged. For instance, profile 4.1.5 fitted *B. licheniformis* while profiles 4.4.3, 2.1.7 and 3.7.7 most probably denoted *B. laterosporus, B. firmus and B. subtilis*, respectively.

Table 2. Frequency of numerical profiles occurrence in relation to the inhibition zone measured for their cell free extract against *S. aureus* ATCC 6538

Profiles generated	occurrence Frequency of each profile	No. of isolates in relation to inhibition zone diameter		
		None	≤ 10 mm.	> 11 mm.
4.1.5	18	3	5	10
3.7.7	6	0	2	4
2.5.2	9	1	3	5
4.4.3	13	12	1	0
2.1.7	12	10	2	0
4.4.5	9	6	1	2
5.3.4	7	4	2	1
0.0.1	7	7	0	0
3.4.5	7	0	2	5
4.7.5	4	0	0	4
4.6.6	4	0	1	3
1.0.3	4	0	2	2
Total	100	43	21	36

4. Discussion

Numerical profiling is not a new approach for the study of bacterial diversity. Griffiths and Lovitt (1980) were the first to use this system while investigating the bacterial diversity of oil storage tanks water bottom. These authors suggested that the system could be used to study bacterial diversity in different ecosystems. Abu Shaqra and Mashni (2006) used the numerical profiling for studying the bacterial diversity of hard gelatine capsules contaminants and proposed the system as a significant tool for establishing the diversity of contaminants in cosmetic and pharmaceutical products. However, methods for studying bacterial diversity in soil are available (Kirk *et al*, 2004), as far as is known, numerical profiling as a mean for establishing the diversity of spore forming bacilli in soil was never used.

Tests used for the characterization of isolates were chosen on the basis of their convenience and ability to establish diversity. For instance, spore formation test and reaction to Gram stain though were carried out, they were excluded because they were of no significance in determining the diversity of *Bacillus* as the vast majority of species gave positive reaction to Gram stain and by most produced endospores (Sneath, 1986).

The best test to be selected for the detection of bacterial diversity in a particular habitat is the one which can differentiate between isolates on equal bases. For clarification, the ideal test should give positive results for 50% of isolates and negative test results for the remaining 50 % (Griffiths and Lovitt, 1980). Figure 1 demonstrates the percentage occurrence of positive characters among our isolates and indicates that none of the tests used was ideal as no single test was capable of 50% differentiation. It is evident that this method relies on stressing the characters of the organism rather than focussing on their names, although in occasions as the case is in this investigation, assigning names was possible.

The diversity of *Bacillus* species in soil varies considerably in terms of numbers and types of isolates. Nishijima *et al.,* (2005) have studied soil samples collected from different locations in Japan and found that number of spore forming bacteria did not differ a lot from one place to another but the types of isolates did. They were able to recover up to 18 different species from one location whereas; only one species was isolated from another. In this work 12 different numerical profiles were generated for the isolates. Had more tests been used in the generation of profiles, diversity could have been higher. In fact addition of tests for the construction of the numerical profiling system merely requires the increase in the number of digits; instead of 3 digits, 4 or 5 digits could be constructed.

The frequency of occurrence of each profile generated is illustrated in table 2. It is clear from this table that profile 4.1.5 was the most frequently encountered, followed by 4.4.3 while isolates labeled with profiles 5.7.5, 4.6.6 and 1.0.3 were the least encountered. These observations are consistent with those made by Kuta *et al.,* (2009) who found that frequency of occurrence among their *Bacillus* soil isolates (identified to species level by conventional methods) varied between 1.9% and 30.8%. The close similarity of results reported herein and those of Kuta *et al.,* (2009) indicated the effectiveness of the numerical profiling system in dealing with soil bacterial diversity as compared to traditional approaches.

Soil is a well known source of microorganisms capable of producing a variety of biologically active metabolites including antibiotics. Table 2 shows that 36% of *Bacillus* isolates recovered from Ajloon soil exhibited effective antagonistic activity against *S. aureus* ATCC 6538. This percentage is not far away from that determined by Kuta *et al.,* (2009) who found that 41.7% of *Bacillus* species isolated from soil samples obtained from 16 refuse dump sites in Minna – Niger were inhibitory against a strain of *S. aureus.*

It is of profound importance to note that isolates with similar profiles did not give 100% exact results in the antimicrobial activity test. This particular observation is of dual significance. First, had isolates with the same profile been with absolute phylogenetic relation and this is clearly not the case, all would have given the same inhibitory activity against the test organism. The lack of this absolute relatedness was expected as tests used were dependent on the biochemical activity and no tests were performed to identify the genotype of isolates. However, simple and limited number of tests was used, numerical profiling system proved to be as good as any conventional approach employed for the study of bacterial diversity. Second, not all isolates with similar profiles gave the same spectrum of activity against *S. aureus*. Out of 18 isolates labeled with the profile 4.1.5, 10 were effective inhibitors while, 5 and 3 isolates were with limited or no antimicrobial activity, respectively.

Statistical significance was found to exist ($p < 0.05$) between each profile and the number of isolates grouped in that profile with respect to the zone of inhibition measured. Table 2 shows that isolates with profiles 4.4.3 and 2.1.7 catered for 13 as well as 12 isolates, respectively, with the majority of these isolates being none inhibitory to the test organism.

Based on the outcome of this work, future study may exempt isolates labeled with profiles that were found to be of poor antimicrobial effect from being screened for inhibitory activity as they are unlikely to exhibit antimicrobial property. It might be thought that the use of a single bacterial species in the antimicrobial study is a draw back in the set up of this investigation, the fact is, this work was not designed to isolate a strain or strains of *Bacillus* with broad spectrum of antimicrobial activity but to demonstrate the usefulness of the profiling system in predicting the antimicrobial potentials of isolates and this was achieved.

5. Conclusion

This investigation has shown for the first time that numerical profiling is a useful technique for studying the diversity of soil spore forming bacteria. It also points out to the possibility of exploiting the same system in the prediction of antibacterial activity of isolates recovered from this extremely diverse habitat.

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