

Characterization and Identification of Some Aerobic Spore-Forming Bacteria Isolated From Saline Habitat, West Coastal Region, Saudi Arabia

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Abstract: Ten isolates of aerobic endospore-forming moderately halophilic bacteria were isolated from saline habitat at the west coastal region near Jeddah. All isolates that were Gram positive, catalase positive and showing different colony morphology and shapes were studied. They were mesophilic, neutralophilic, with temperature range 20-40°C and pH range 7-9. The isolates were separating into two distinct groups facultative anaerobic strictly aerobic. One isolate was identified as *Paenibacillus dendritiformis*, two isolates as *Bacillus oleronius*, two isolates as *P. alvei*, three isolates belong to *B. subtilis* and *B. atrophaeus*, and finally two isolates was identified as *Bacillus* sp. Furthermore, two aerobic endospore-forming cocci, isolated from salt-march soil in Germany were tested for their taxonomical status and used as reference isolates and these isolates belong to the species *Halobacillus halophilus*. Chemotaxonomic characteristics represented by cell wall analysis and fatty acid profiles of some selected isolates were studied to determine the differences between species.

Keywords: *Halobacillus*, *Bacillus*, spore, mesophilic, physiological, morphological

I. Introduction

Aerobic spore-forming bacteria represent a major microflora in many natural biotopes and play an important role in ecosystem development. These bacteria are able to transform many chemical compounds including industrial pollutants. In the past, most of strains belong to the heterogeneous group of aerobic, endospore-forming, Gram-positive bacteria and were classified in genus *Bacillus* (Berkeley and Goodfellow, 1981; Claus and Berkeley, 1986) and *Sporosarcina* (Claus *et al.*, 1983, 1984). However, later this group became larger and included other genera such as *Amphibacillus* and *Sulfobacillus* (Holt *et al.*, 1994). Classification of genus *Bacillus* that included heterogeneous isolates with similar phenotypic characteristics, nutrition requirements, and 33.8- 60.4% guanine and cytosine ratio (Fahmy *et al.*, 1985) is very important. According to the phenotypic classification, 368 strains of the genus *Bacillus* were classified into 79 different taxonomic groups (Priest *et al.*, 1988). Moreover, recent phenotypic and evolutionary phylogenetic taxonomic studies resulted in classifying a number of new genera into this group including *Filobacillus* (Schlesner *et al.*, 2001), *Geobacillus*, *Gracilibacillus* and *Salibacillus* (Wain *et al.*, 1999), *Halobacillus* (Spring *et al.*, 1996) *Jeotgalibacillus* and *Marinibacillus* (Yoon *et al.*, 2001b), *Ureibacillus* (Fortina *et al.*, 2001), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Paenibacillus* (Ash *et al.*, 1993), *Aneurinibacillus*, (Shida *et al.*, 1996) and *Brevibacillus* (Shida *et al.*, 1996). The genus *Sporosarcina* is characterized by spherical bacteria arranged into groups of four, in cubes consisting of eight cells, or other multiple of four arrangements and this genus contained two species, *S. ureae* and *S. halophila* (Claus and Fahmy, 1986) but based on a study of the evolutionary traits, *S. halophila* was re-classified under the genus *Halobacillus* (Spring *et al.*, 1996). In 2001, *S. aquimarina* was isolated from seawater in Korea (Yoon, *et al.* 2001a) and was added to genus *Sporosarcina* based on chemotaxonomy and evolutionary which based on amino acids analysis and 16S rRNA sequence data (Busse *et al.* 1999). Three *Bacillus* isolates were added to genus *Sporosarcina*, *S. globispora*, *S. pasteurii* and *S. psychrophila* (Yoon *et al.* 2001a). In addition, *S. macmurdoensis* was added to the previous genus (Reddy *et al.*, 2003). Then, the genus *Sporosarcina* was re-classified under the *Bacillales* and *Planococcaceae* (Garrity *et al.*, 2002, 2004). Therefore, the investigation of these micro-organisms is highly important for microbiologists dealing with ecological studies and environment protection. The aim of this study was isolation and classification of some spore forming bacterial isolates, obtained from marine habitats, Jeddah, Saudi Arabia.

II. Material And Methods

Bacterial isolation

Ten bacterial isolates from a saline habitat at the west coastal region near Jeddah, were obtained using a dilution plating technique on modified Tryptone Soy Agar (CM 0131 Oxoid), prepared with artificial seawater (Yoon *et al.*, 2001a). *Bacillus megaterium* (DSM 90) and *Halobacillus halophilus* (DSM 2266) from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, were used as reference strains.

Morphological and physiological characterization:

The morphological characters on modified Tryptone Soy Agar at 30°C were determined (Claus and Berkely, 1986; Smibert and Krieg, 1994). Cell morphology and motility were observed by phase-contrast microscopy while Gram staining was done as described by Salle (1961) and Malik (1992). Optimum temperature and pH, temperature and pH ranges, growth under anaerobic conditions in an anaerobic jar using Oxoid gas generating kit- BR 38 (Quesada *et al.*, 1984), and tolerance to different concentrations of sodium chloride on nutrient agar supplemented with 2-20% NaCl (w/v) were investigated (Spring *et al.*, 1996). The oxidase activity (Liu and Jurtschuk, 1986) and catalase activity (Whittenbury 1964, Haynes 1972) were conducted. Hydrolysis of Starch, Casein, Tyrosine and Cellulose (Lanyi, 1987) in addition to other biochemical tests using API 20E (bio Merieux) were examined (Logan and Berkeley 1981).

Chemotaxonomic characterization:

The chemical analysis of the cell wall was carried out by the methods described by Atrih *et al.* (1999) and Mckerrow *et al.* (2000). The peptidoglycan structure was determined using the method of Harper and Davis (1979). Fatty acids were extracted and prepared as described by Sasser (2001). The fatty acid analysis and identification were completed by Nelson Laboratories according to the instructions of the Microbial Identification System (MIDI).

III. Results

After incubation on modified Tryptone Soy Agar at 30°C, ten isolates were obtained and named SAJ5, SAJ7, SAJ8, SAJ11, SAJ12, SAJ13, SAJ14, SAJ16, SAJ17 and SAJ19. All isolates showed different colony morphology and all cells were straight rods, mono or diplo bacilli. Some of the isolates had short chains of cells and some of them had palisade arrangement (Figure 1). All isolates were Gram-positive, motile and spore-formers while some of the isolates had swollen sporangia or had both swollen and non swollen sporangia in the same microscopic field of the pure culture (Figure 2). The spores were located in different positions, centrally, sub-terminally or terminally (Figure 3).

All isolates were mesophilic, neutralophilic, with temperature range 20 -40°C, and pH range 7- 9. Therefore, the temperature used for all tests was 30°C, and pH 7. The isolates tolerate 5-15% sodium chloride and some isolates could withstand heating till 95°C for 15 min. Positive results of all the isolates were positive for catalase, oxidase, starch hydrolysis and tyrosine decomposition. Negative results were for lysis by 3% KOH, hydrolysis of cellulose, phenylalanine deaminase, VP test, acid production from raffinose and arginine dihydrolase, Lysine decarboxylase, Ornithine decarboxylase, Tryptophane desaminase, indole and H₂S production. Different results were recorded for gelatin and casein hydrolysis, nitrate reduction, Acid production from D-Glucose (Table 1).

According to the ability to grow under anaerobic conditions, the isolates could be separated into two groups. Group 1 represents those isolates that grow under anaerobic conditions, isolates SAJ5, SAJ3 and SAJ19. This group was further subdivided into two subgroups according to pH range, and growth on nutrient agar containing MgCl₂. Group 2 includes those which were strictly aerobic isolates SAJ7, SAJ8, SAJ11, SAJ12, SAJ14, SAJ16 and SAJ17. Further classification of group 2 according to temperature range, heat resistance of spore, and sodium chloride tolerance resulted in separating these seven isolates into six subgroups. By applying the described biochemical tests and according to the results of the other routine phenotypic tests this classification is confirmed. Using the taxonomical key for identification of this group of aerobic endospore-forming bacteria (Reva *et al.*, 2001), with its new proposed and established genera and species, eight isolates from the present rod-shaped ones could be identified. The isolate SAJ5 identified as *Paenibacillus dendritiformis*. Two isolates SAJ7 and SAJ8 were identified as *Bacillus oleronius*, and other two isolates SAJ13 and SAJ19 as *Paenibacillus alvei*. The last three isolates SAJ11, SAJ12 and SAJ17 were identified as isolates belonging to *B. subtilis* and *P. kobensis*, according to the absence or the presence of swollen sporangia and using the keys for identification of the typical *Bacillus* species. For isolates SAJ14 and SAJ16, they were recorded as new isolates because they were not identical to any of the species reported in the used keys.

Identification of the isolates SAJ5, SAJ14, SAJ16 and SAJ-17 in addition to the reference type strain *B. megaterium* (DSMZ 90) was confirmed using the analysis of their cell walls. The results revealed that the murein type of the reference type strain of *B. megaterium* DSMZ 90 showed murein type (A4 γ). Isolates SAJ14 and SAJ16 showed the same murein type as that of *B. megaterium* (A4 γ) while, isolate SAJ17 had the same murein type with an additional amino acid (Asp). The murein type of isolate SAJ 5 was like to that of *H. halophilus* (Orn-Asp: A4 β).

Moreover, fatty acids were extracted from the isolates SAJ5, SAJ14, and SAJ17 and the results of this analysis showed agreement with the identification made in this study using the identification keys. However, the analysis of isolate SAJ14 failed to determine the type of the isolate according to all listed types in (MIDI), which agree with obtained results in this study based on the used identification key.

IV. Discussion

Aerobic spore-forming bacteria represent by genus *Bacillus* are a group of bacteria that occupy diverse ecological systems and have been isolated from various habitats (Logan and De Vos, 2009). In recent years, there are 751 species in 73 *Bacillus*-related genera found in the world (Liu *et al.*, 2015). Many of these names are not present in the previous keys for identification of this group. This situation causes difficulties in identification of any aerobic spore-former. This problem of taxonomical status of this group was very appealing. Especially that some researchers use different set of morphological and physiological characteristics to identification new genera or species (Reva *et al.*, 2001). Therefore, to have a well classification and identification of this bacterial group, the chemotaxonomic methods were applied, which determined based on similarities in the chemical structure of certain compounds. One of chemotaxonomic techniques is the chemical analysis of the bacterial cell wall and identifies the muerin type which is widely used to identify and classify several species of bacteria (El-Rahman *et al.*, 2002, Albuquerque *et al.*, 2008, Madhaiyan *et al.*, 2010, Dunlap *et al.*, 2015). Furthermore, the analysis of the fatty acids is used in the identification and classification of this group. Typically, this analysis is conducted by either gas-liquid chromatography (GLC) (Ivanova *et al.*, 2003) or by gas chromatography (Gugger *et al.*, 2002; Ajithkumar *et al.*, 2003). The results of the chromatography analysis can be quantitatively analyzed and electronically stored in a computer for further comparison (Stead *et al.*, 1992). Therefore; the identification of the indeterminate bacterial isolates can be conducted by comparing these isolates against the commercial (MIDI). This microbial identification system is widely used to identify unknown bacteria (Palmisano *et al.*, 2001, Logan *et al.*, 2002, Groudieva *et al.*, 2003, Balca' zar *et al.*, 2010, Vaz-Moreira *et al.*, 2012, Reddy *et al.*, 2015). Finally, to confirm the identification of the isolates already denoted to particular species, or the unknowns, further experimental analyses have to take place, such as protein profiles, G + C % and hybridization with specific species using DNA and RNA. The results revealed that the murein type of *B. megaterium* DSMZ 90 is A4 γ and the same results was obtained by Bricas *et al.* (1967) and Schleifer and Kandler (1972). Moreover, based on the high agreements between our results and the analysis conducted by Nelson labs, isolate SAJ14 can be classified as new specie. However, further investigation and more experiments need to be carried out to confirm the identification of this isolate.

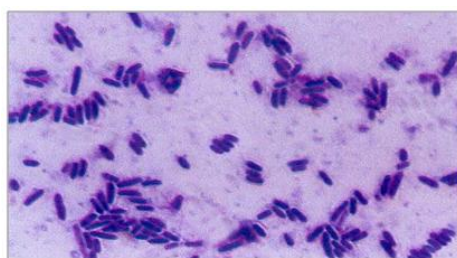


Figure 1. Light microscope (x1000) of rods and palisade shape of the isolate SAJ14

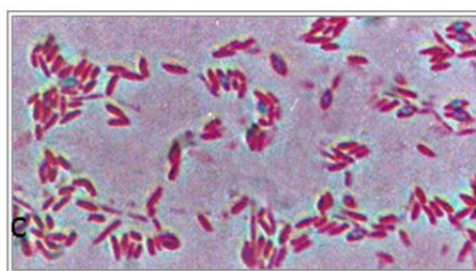


Figure 2. Light microscope (x1000) of endospores and vegetative cells of the isolate SAJ8

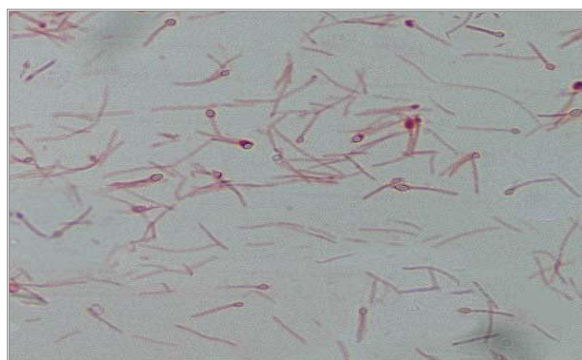


Figure3. Light microscope (x1000) of terminal endospores and vegetative cells of the isolate SAJ11.

Table1. Differential phenotypic characteristics of the tested bacterial isolates

Character tested	SAJ-5	SAJ-7	SAJ-8	SAJ-11	SAJ-12	SAJ-13	SAJ-14	SAJ-16	SAJ-17	SAJ-19
Colony color	Orange			Cream		Orange	Cream	Light orange	Light yellow	Cream
Cell morphology	R (SC)	R (DB)	R (PA)	R (DB)	R (DB)	R (PA)	R (PA)	R (SC)	R (SC)	R (DB)
Endospore location	C	ST	C/ST	T	T	C/ST	C/ST	C/ST	T	C
Swollen sporangia	S	S	S	S/US	S/US	S/US	S	S	S/US	S
Growth conditions										
Anaerobic growth	+	-	-	-	-	+	-	-	-	+
Growth temperature range (°C)	10-40	15-40	10-40	10-40	15-40	10-40	20-45	10-40	10-40	10-40
pH range	6-9	6-9	6-9	6-10	6-10.5	6-10.5	5-9	6-10.5	6-10.5	6-10.5
NaCl (%) range	2-15	2-5	2-5	3-5	2-7	3-15	2-10	2-7	2-7	3-15
Hydrolysis of:										
Gelatin	+	+	+	-	-	+	-	-	-	+
Casein	+	-	-	-	-	+	-	-	-	+
Nitrate reduction	-	-	-	+	+	-	-	-	+	-
Acid production from D-Glucose	±	-	-	±	±	±	-	-	±	-

*R: rods; SC: Short chains; PA: Palisade arrangement, DB: Diplobacilli; T: Terminal spore;

C: Central, ST: Sub-terminal spore, S: Swollen; US: Unswollen; +: Positive; ±: Weakly positive; -: Negative

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