Isolation of endospore-forming bacteria from marine sources

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Introduction

The production of endospores by several bacterial genus provides them the capability to survive adverse environmental conditions, and ensures a wide dispersion through different habitats. Most of endospore-forming genera have been placed in the section 13 of the *Bergey's Manual of Systematic Bacteriology* (Sneath et al., 1986) based on this trait. The physiological diversity among endospore-forming bacteria includes a variety of metabolic process and products which contribute to the survival in different environments and have clinical and industrial importance.

At the end of 1992, the endospore-formers were allocated to 13 validly published genera which, taken together, form a group of some morphological, physiological and genetic diversity (Berkeley and Ali, 1994). These genera are *Alicyclobacillus, Amphibacillus, Bacillus, Clostridium, Desulfotomaculum, Oscillospira, Pasteuria, Sporohalobacter, Sporolactobacillus, Sporosarcina, Sulfobacillus, Syntrophospora,* and *Thermoactinomyces*. Controversy exists today about the validity of some species and genera. In the last years, new species have been added to some of this genera.

In marine environments, organisms remarkably distinct from the terrestrial ones can be found. Aerobic endospore-forming bacteria in the marsh and open ocean exhibit red, yellow and pink pigments. Evidence for the differences between both environments in seen in *Sporosarcina*. *Sporosarcina ureae* can be isolated from garden and field soils (Gibson, 1935), sea water (Wood, 1946), and soil from the base of the trees where dogs

have urinated (Pregerson, 1973). *Sporosarcina halophila* has been isolated exclusively from salt marsh soil in Germany (Claus et al., 1983).

Work done by Singer and Leadbetter (year?) resulted in the isolation of pigmented aerobic spore-forming bacteria from Sippewisset salt marsh. They show that the isolation required media containing sea water and that less than 1% of the bacterial population were sporeformers. The objective of this study is to isolate and compare aerobic and anaerobic population of endospore-forming bacteria from marine sources.

Materials and Methods

Sample-Samples were collected from water and marine sediments of Sippewisset salt marsh, MBL Beach, Garbage Beach, Eel Pound, and aquarium ramp at Woods Hole, Massachusetts. These were processed as integrated samples according to the original source (Table 1).

Table 1. Origin and composition of integrated samples.

Integrated Sample	Source	Components
EP1	Eel Pound	sea water and sediments
GB1	Garbage Beach	sea water and sediments
MBL-1	MBL Beach	sediment under water
MBL-2	MBL Beach	sea water and sediments
MBL-3	MBL Beach	sea water and sediments
R1	Aquarium Ramp	sea water and sediments
SM-I	Sippewisset Salt Marsh	dry sand
SM-II	Sippewisset Salt Marsh	soil and water near to bridge
SM-III	Sippewisset Salt Marsh	sludge and grass soil
SM-IV	Sippewisset Salt Marsh	water with colloidal sulfate
SM-V	Sippewisset Salt Marsh	mats

Culture- Except where specify other way, aerobic cultures were incubated at 30°C for 48 hr., and anaerobic cultures at 25°C for 7 days. One gram of sediment (or 1 mL of water) from each sample was diluted 10^{-2} to 10^{-5} in sterile sea water. Each diluted sample was pasteurized (heat 10 min at 80°C and cool rapidly on ice). Dilutions 10^{-2} were cultured aerobic and anaerobically on different media (Table 2). Dilutions 10^{-3} to 10^{-5} were cultivated aerobically on SWC and NA-SW. All plates were incubated aerobic (except YEG-SW) and anaerobically. After incubation, colonies were enumerated from SWC,

NA-SW and YEG-SW plates to estimate population. Different colonies were isolated to generate pure culture and identify their genus. Identification of genus was based on microscopic examination, colony morphology, several biochemical tests and growth conditions. Cultures were stored at 4°C in NA-SW plates.

Table 2. Media used for isolation of different endospore-forming bacteria.

Media[a]	Propose
Sea Water Complete (SWC) [peptone, 5 g; glycerol 3 mL; agar, 15 g]	Isolation and enumeration of endospore-forming bacteria
Glucose-Yeast Extract-Peptone (GYE) [glucose, 10 g; yeast extract, 5 g; peptone, 5 g; agar, 15 g]	Isolation of *Sporolactobacillus*
Medium for *S. ureae* (Sur) [tryptic soy broth, 27.5 g; yeast extract, 5 g; glucose, 5 g; agar, 15 g; pH 8.1. Urea 1% after sterilization.]	Isolation of *Sporosarcina ureae*
Nutrient Agar (NA-SW) [peptone, 5 g; beef extract 8 g; agar, 15 g]	Isolation and enumeration of endospore-forming bacteria (with special interest on *Sporosarcina halophila*)
Yeast Extract-Glucose (YEG-SW) [glucose, 10 g; yeast extract, 5 g; cysteine, 0.05%; agar, 15 g]	Isolation and enumeration of anaerobic endospore-forming bacteria

[a] made in 1 liter of sea water:destillated water (3:1).

Microscopy examination-The microscopic examination was done using phase microscopy to determine motility and natural shape of the cells; and light microscopy for Gram staining.

Colony morphology-Colonies were described on bases of intracellular pigment production and their form, elevation and margin (Smibert and Krieg, 1994).

Biochemical Tests-The physiological characterization of the isolates was made through the catalase and oxidase test, and hydrolysis of starch and casein. For the catalase test,

a small portion of fresh culture for every isolate was added to one drop of hydrogen peroxide to observe the evolution of bubbles as positive result. The oxidase test was done adding the oxidase reagent (Difco Co.; Detroit, MI) on fresh colonies of the isolates. The production of purple color in the colony was interpreted as positive result while no change in color was considered as negative reaction. The hydrolysis of starch was assayed on fresh cultures growing on plates containing starch (0.2% w/v) and agar (1.5% w/v) on sea water:destiled water (3:1). Gram iodine was added to the colonies to detect clearing zones around the bacteria as positive reaction for this test. The hydrolysis of casein was determined for cultures on casein sea water agar (casein, 50 g; agar 15 g; sea water, 750 mL; distillated water; 250 mL). After incubation, clearing zone around the colony was considered positive reaction.

Growth conditions-Oxygen, temperature, light and nutritional were evaluated in this study. Isolates were plating on NA-SW and incubate aerobic and anaerobically at 25, 30, 37, 42 and 55°C to determine the oxygen and temperature required for growth. Anaerobic incubation was done in anaerobic chamber (25°C) and Gas Pak jars (30, 37, 42 and 55 °C). Light effect was tested by the inoculation of isolates in NA-SW and their incubation in the dark and exposed to light. Isolates were cultured in SWC (a minimal media), NA-SW (complex media) and NA (non sea water) plates to compare the nutritional preferences on bases of growth. In all cases, the amount of growth was used as qualitative measurement.

In situ hybridization-Several samples were ***subjected*** to *in situ* hybridization using Low G + C and universal probes to detect endospore-forming bacteria. One rod and one cocci isolated were used as control. The procedure was done as describe by Nierzwicki-Bauer (1996).

Results and Discussion

Endospore-forming bacteria can be found in all environments. They were obtained from marine sources in every sample of this study. This organisms produce endospore that ensure their survival in adverse environments. The production of endospore by several bacterial genus make us to suppose that they could be found in high quantity and equally distributed in nature. In this study, the population of endosporers was variable among places evaluated and incubation conditions (Table 3).

Table 3. Population of endospore-forming bacteria from marine sources.

Sample	Aerobic Isolation[a]		Anaerobic Isolation[a]		
	SWC	NA-SW	YEG-SW	SWC[a]	NA-SW[b]
Eel Pound	3×10^8	1×10^8	1×10^3	$> 10^3$	$> 10^3$
Garbage Beach	3×10^8	2×10^7	100	6×10^3	157
MBL Beach 1	$> 10^8$	5×10^8	6×10^3	5×10^3	126
MBL Beach 2	3×10^8	5×10^7	0	0	0
MBL Beach 3	$> 10^8$	$> 10^8$	0	0	0
Ramp Aquarium	1×10^8	2×10^6	0	5×10^3	$> 10^3$
Salt Marsh I	1×10^8	1×10^7	0	4×10^3	148
Salt Marsh II	3×10^8	3×10^8	0	4×10^3	$> 10^3$
Salt Marsh III	3×10^8	2×10^8	0	5×10^3	$> 10^3$
Salt Marsh IV	2×10^8	1×10^8	0	4×10^3	$> 10^3$
Salt Marsh V	2×10^8	2×10^8	0	4×10^3	$> 10^3$

[a] cell/mL or g
[b] pinpoint colorless colonies

Aerobic population was obtained in range in 10^7 to more than 10^8 cells/g or mL; while the anaerobic was obtained mainly in range less than 1600 cell/g or mL. This data suggests that endospores grown aerobically predominate in the environments studied.

The endospores seem to be exposed to different stress or selective pressures that influence in their survival and distribution.

The marine endosporer differs, at least, from soil ones on the production of intense intracellular pigments. The isolation of pigmented endospore-forming bacteria was more diverse on NA-SW than SWC (Table 4).

Table 4. Pigments produced from aerobic isolates.

Sample	Pigments produced	
	SWC	NA-SW
Eel Pound	white	orange, pink, white, yellow
Garbage Beach	white	orange, pink, white, yellow
MBL Beach 1	white	orange, pink, white, yellow
MBL Beach 2	white	pink, yellow
MBL Beach 3	white	white
Ramp Aquarium	white	pink
Salt Marsh I	white	yellow
Salt Marsh II	white	pink, white, yellow
Salt Marsh III	white, yellow	orange, pink, white, yellow
Salt Marsh IV	white	orange, pink, yellow
Salt Marsh V	white, yellow	orange, pink, white, yellow

Nutrient Agar-Sea water media is richer than SWC because it contains beef extract (Table 2). In both media, bacteria were isolated in order 10^6 cells/g or mL (Table 3). Nutrient Agar (containing $MgCl_2$ and NaCl) and Marine Agar are recommended for the isolation of *Sporosarcina halophila* (Claus et al., 1983). Small pigmented colonies were accidentally observed seven days after inoculation when clean the lab up. Endospore-forming bacteria well pigmented grow with lower level pigmentation on SWC. Also, a

higher pigment diversity of colonies was obtained from sources rich on organic matter as salt marsh, Eel pound and Garbage Beach (Table 4). This evidence suggest that pigmented isolates seem to have more complex nutritional requirements than unpigmented ones to growth fast and some natural environments can provides it.

The oxygen availability seems to have effect on pigment production by endospore-forming bacteria. Table 5 shows that more pigment variety was obtained under aerobic incubation. This table represent the number of bacterial isolates but not the relative abundance of each pigmented group in the sample.

Table 5. Production of pigment under different oxygen condition of isolation.

Oxygen Condition for Isolation	Pigment Produced	Number of Isolates
Aerobic	Orange	21
	Pink	4
	White	44
	Yellow	9
	TOTAL	71
Anaerobic	Orange-Brown[a]	2
	White	11
	TOTAL	13

[a] produced only by old colonies (more than 7 days incubation)

Pigmented colonies were more abundant and morphologically similar than unpigmented ones for each sample in every dilution (Data not shown). Most of the isolates demonstrated to be facultatively anaerobic when they was cultured and incubated under atmosphere contrary to the initial isolation (Table 6).

Table 6. Some biochemical and morphological traits of isolates.

Trait	Aerobic Isolates[a]					Anaerobic
	Orange	Pink	White	Yellow	Total	Isolates[a]
Shape-cocci	15	0	18	14	16	0
Shape-rod	85	100	82	86	84	100
Catalase +	85	0	93	100	87	0
Catalase -	15	100	7	0	13	100
Motility	70	100	34	56	48	62
Facultatively Anaerobic	81	75	36	100	42	100
Casein +	48	50	35	44	40	0
Casein -	52	50	65	56	60	100
Oxidase +	60	75	59	78	63	ND[b]
Oxidase -	40	25	41	22	37	ND
Starch +	71	75	70	44	68	40
Starch -	14	25	30	56	28	60

[a] percentage of isolates
[b] not determined

The aerobic pigmented isolates exhibited low pigmentation when were cultivated under anaerobic conditions (Table 7). The anaerobic pigmented isolates failed to produce pigment when they are grown aerobically (Table 7). The following two cases support our idea of endospore-forming bacteria require oxygen to produce pigment. The pigmentation of *Halobacterium*, bacterioruberins, decreases from purple to red or orange when cells are cultivated anaerobically (Brock et al., 1994). The non-sulfur purple bacteria only produce pigment, bacteriochlorophyll, aerobically in either the light or the dark (Brock et al., 1994).

Table 7. Qualitative pigmentation level under different growth conditions.

Growth	Pigment Level[a]

Condition	Aerobic Isolates	Anaerobic Isolates
Aerobic Incubation	High	Low
Anaerobic Incubation	Low	High
SWC	Low	Low
Non Sea water media (NA)	Low	Low
Dark	High	Low
Light	Low	Low
Temperature 25-37°C	High	High
Temperature > 37°C	Low	Low

[a] comparison made with original isolation conditions (cultivation on NA-SW at 30°C for 48 hr.)

The light effect was also considerated in this study. Our bacteria decrease on pigment intensity when they are grown in the dark (Table 7). The pigment seems to be activated or induced by light as been reported for photochromogenous species of *Mycobacterium* (Brock et al., 1994). Previously, the carotenoid pigments has been proposed as photoprotective agent in photosynthetic bacteria and *Deinococcus* (Brock et al., 1994). The pigment protects the cell against harmful light, ultraviolet radiation and desiccation (Brock et al., 1994). The bright light can be often harmful to cells in that it causes various photooxidation reaction that can actually lead to the destruction of chlorophyll and the photosynthetic apparatus itself (Brock et al., 1994). The carotene absorb much of this harmful light and thus provide a shield for the light sensitive chlorophyll (Brock et al., 1994). **By other side,** light has been shown to maintain the viability of culture of *Halobacterium* incubated anaerobically in the absence of organic energy source (Brock et al., 1994). Since endospore-forming bacteria in marine sources are exposed to the light, as well as photosynthetic bacteria, the photoprotective role of carotenoids is of obvious advantage.

In bases of the colors exhibited by endospore-forming bacteria colonies (orange, pink and yellow) probably they have carotenoid pigments. They are the most widespread accessory pigments. They are present in *Staphylococcus aureus, Mycobacterium, Deinococcus,* mycoplasmas and photosynthetic, halophilic, green and non-sulfur purple bacteria. They absorb light in the blue range of the spectrum to exhibit yellowish, reddish, brownish and greenish colors. They protect photosynthetic bacteria from harmful light, some esophilic bacteria from high temperature, *S. aureus* from phagocytic cells, *Deinococcus* from UV radiation, mycoplasma from osmotic lysis and phototrophic and airborne bacteria from toxic form of oxygen (Brock et al., 1994). Also they are involved in the metabolism of halophilic Archea and photosynthetic bacteria.

The application of pigment production as classification trait was evaluated for endospore-forming bacteria from marine environments. Microscopic examination and several biochemical tests were done to the isolates. Isolates were grouped in bases of their pigmentation in order to compare several morphological and biochemical characteristics (Table 6). This analysis show that most of the isolate share the main traits for endospore-forming bacteria: catalase and motility. But the strains into each pigmented group have not reaction in common for shape, oxidase, starch nor casein. With the currently schemes, the pigment production by endospore-forming bacteria is not useful for their identification and taxonomical classification. Similar idea has been adopted for purple and green bacteria that also have carotenoid pigments. Pigment production has been correlated to taxonomic group in *Mycobacterium* and by now it seems to be useful for speciation.

Our data suggest the presence of at less two genus of endosporers in the studied places: *Sporosarcina* and *Bacillus* (Figure 1). *Sporosarcina* is the unique cocci-shaped endospore former bacteria. *Bacillus* is a rod-shaped endosporer bacteria and

facultatively anaerobic. Although, more examination is needed to identify definitely each isolate and lock for the possible new taxons.

In comparison to previous work in this specific topic, this research confirm the isolation of high population of aerobic endosporer from marine environments but is contrary to the requirement of sea water for growth and to the use a minimal media for isolation purpose (Singer and Leadbetter, 19??).

By other way, fluorescence *in situ* hybridization was useful applied for the detection of endosporer from marine samples (Table 8). Samples pausterized from salt marsh, MBL Beach, Garbage Beach, Eel Pound and Aquarium Ramp showed fluorescence rod-shaped bacteria Low G + C either universal probes. Rod and cocci endosporing isolate from marine environment were used as control with Low G + C probe. Only the rod was detected. It suggests that *Sporosarcina*, the endosporing cocci, is refractile to the with Low G + C probe.

Table 8. *In situ* Hybridization assay to marine samples.

Sample	Low G + C Probe	Universal Probe
Rod isolate	+	ND[a]
Cocci isolate	-	ND
Mix EP, GB and R	+	ND
MBL Beach 1	+	ND
MBL Beach 2 and 3	-	ND
Salt Marsh I	+	+
Salt Marsh II	+	+
Salt Marsh III	+	+
Salt Marsh IV	+	-
Salt Marsh V	-	+

[a] Not Determined

Study the pigment production by endospore-forming bacteria from marine environments would allow us to understand their role and explore possible applications.

Molecular biology and spectrophotometrical techniques would be used to research physiological and genetics aspects of the endospore-forming bacteria.

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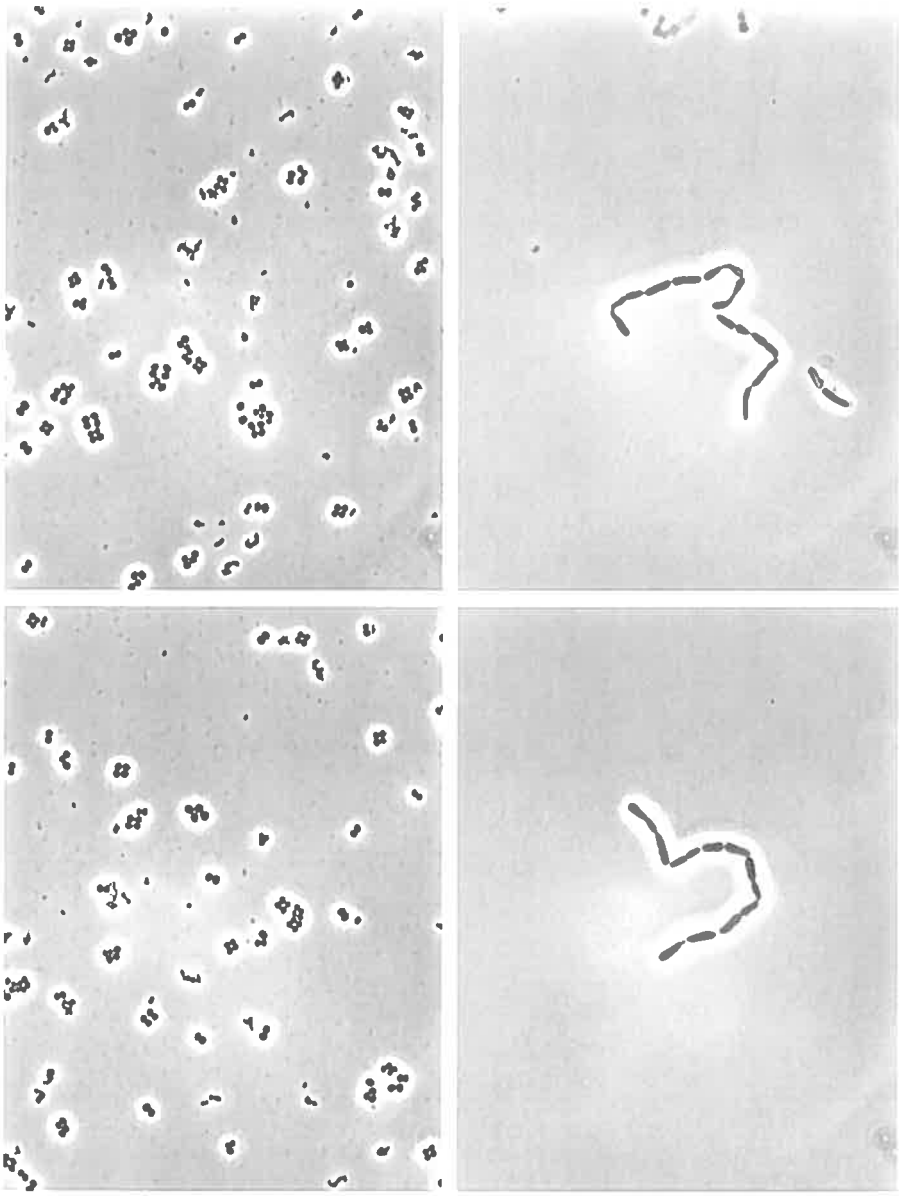

Figure 1-Phase contrast (100X) photomycrograph of aerobic isolates from marine environments in wet mount. Cocci tetrads of *Sporosarcina* (left) and rod chains of *Bacillus* (right).