

# Sand Beach Bacteria: Enumeration and Characterization

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Bacteria in the water-saturated sand of a relatively unpolluted sand beach were enumerated by direct microscope and viable counting. The number of interstitial bacteria was estimated to be a significant fraction of the total number of bacteria present. Three hundred sixty-two strains were isolated and submitted to cultural and biochemical tests. Fermentational abilities and the production of indole suggested that a significant number of these bacteria were symbiotically associated with resident metazoans.

Bacteria are considered to be an important component of the sand beach community (5-7, 12, 14). The productivity of the sand beach is ultimately limited by nutrient input. From laboratory studies, it appears that nutrients (carbon) pass first through the bacterial community and then into the protozoan and metazoan community (12). To initiate studies on sand beach bacteria in Lebanon, a tideless, fully exposed marine beach was chosen: Sindbad beach, 30 km south of Beirut. The beach has no obvious signs of pollution. Small recreational swimming areas are located on the north and south quarters of its 1.5-km length. In the center part, samples were taken repeatedly, to enumerate bacteria and also to isolate several hundred individual strains to determine their biochemical, morphological, and cultural characteristics.

## MATERIALS AND METHODS

**Enumeration of bacteria in sand.** The total number of bacteria per gram of sand was obtained by: (i) collecting a small sand sample in the field (kept chilled until return to the laboratory); (ii) aseptically adding 6 to 7 g of the wet sand to a pretared 18- by 250-mm sterile screw-cap tube for obtaining the wet weight of sample; (iii) adding sterile 0.1% peptone (Difco) in sea water or distilled water to the 5.0-ml mark of the tube; (iv) vortex shaking for 60 s (longer shaking did not increase the number of bacteria detected); and (v) taking samples from this tube for further dilution and plating on peptone-yeast extract medium (PYE). PYE contained: peptone (Difco), 0.5%; yeast extract, 0.05%; and agar (Difco), 1.5% made with either sea or distilled water. Other media (increasing or decreasing the peptone concentration

with the exclusion of yeast extract, or replacing the yeast extract with glucose or phosphate) supported the growth of fewer bacteria. Interstitial bacteria (not attached to sand grains) were collected with an interstitial water sample (13) which had been thoroughly rinsed with the sample. For enumeration, 1, 5, or 25 ml of the interstitial water was then suctioned through HA-Millipore membranes (pore size, 0.45  $\mu$ m, in Millipore field monitors). PYE broth was then aseptically added to the absorbant pad below the membrane. All incubations were done at room temperature (25 C). The number of colonies on PYE agar plates were counted after 4 days of incubation, and those on Millipore membranes were counted after 36 h of incubation, the latter with the aid of a binocular dissecting microscope. Total (direct microscope) counts were made from untreated interstitial water and sand samples that had been shaken in 0.004 N NaOH. Samples of either were suctioned through Millipore membranes, and 2% erythrosin in 5% aqueous phenol was then added to the absorbant pad to fix and stain the bacteria present on the membrane. After removal of excess stain, the bacteria retained on the filters were counted with the aid of an oil immersion lens.

**Sites.** During 1971 the wave wash zone (WWZ) was sampled at the highest point that the waves kept the sand saturated with water. Also, the water table (WT) was sampled at the point inward from the WWZ where there was 40 cm of sand covering the water table. The WT was usually 3 to 6 m from the WWZ. In 1972, samples were taken from the WWZ, 10 and 20 m inwards on a transect perpendicular to it.

**Bacterial isolates.** All pure cultures were obtained by repetitive streaking on PYE-sea water agar plates and were stored on PYE-sea water agar slants at 4 C.

**Biochemical and cultural determinations.** Sugar fermentation was determined by the method of Hugh and Leifson (9); presence of deoxyribonuclease was determined by the method of Lachica et al. (11); hydrolysis of gelatin and starch was determined as suggested by Skerman (16); the Voges-Proskauer

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reaction was determined by the method of Barrit (1); indole production, motility test, catalase, and oxidase tests were determined by the methods of Skerman (16); and the flagella stain was done by the Leifson procedure (4). Sea water was used to prepare all media except for the Hugh-Leifson medium, which contained 3.0% NaCl, 0.3%  $K_2HPO_4$ , and 0.1%  $MgSO_4$  in place of sea water.

The ability to grow aerobically on single carbon sources was tested by a modification of the method of Stanier et al. (17), in which sea water was used in place of distilled water. Control plates included no added carbon source besides agar and whatever soluble carbon was present in the sea water. No growth was observed on these plates. The ability to grow at various temperatures (50, 37, 25, 15, 5, and 0 C) and at various pH values (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) was checked on PYE-sea water. All test plates were inoculated by the replica plate method from normal PYE-sea water agar master plates.

## RESULTS AND DISCUSSION

The number of bacteria per milliliter of interstitial water accounted for 22 to 46% of the total number of bacteria per gram of wet sand (Fig. 1). The determination of the total number of bacteria in the interstitial water did not involve shaking, and therefore aggregates may have been counted as one cell. Therefore, this number may be underestimated and could be twice as high (8). The numbers of viable bacteria were  $10^{-4}$  that of total bacteria (Fig. 1). These results suggest that a large proportion of the bacteria active in the ecosystem are not grown in the culture medium and indicate the need for further studies of the efficacy of various nonselective media for isolation and enumeration of these bacteria. The numbers of viable bacteria per milliliter of interstitial water were lower than the numbers of viable bacteria per gram of wet sand. The latter was determined by a method involving shaking; the former did not. Thus, both the total (direct microscope count) and viable numbers of bacteria per milliliter of interstitial water were probably representative of the number of bacterial aggregates, whereas the numbers of bacteria per gram of wet sand (direct and viable counts) were probably more indicative of the number of individual cells.

Microscope study by simple (18) and fluorescent staining (2) revealed no bacteria attached to the sand grains in repeated attempts.

Table 1 shows that most of the viable bacteria were evenly distributed in the water-saturated zones of the WWZ and WT. The number of viable bacteria varied by one order of magnitude. It increased 10-fold during a local storm. Marine bacteria seemed to be predominant in

the samples, with an exception of the sample taken at on August 19, i.e. higher numbers of bacteria were grown on the medium containing sea water.

In the spring and summer of 1971, 362 randomly chosen isolates were cultured and maintained as stock cultures for characterization. Table 2 shows the distribution into six arbitrary, physiological groups based on the ability to ferment various sugars. Group 1 isolates were versatile fermenters and comprised only 17% of the isolates; in group 2, the number of isolates capable of limited fermentational versatility was also small, comprising only 23% of the

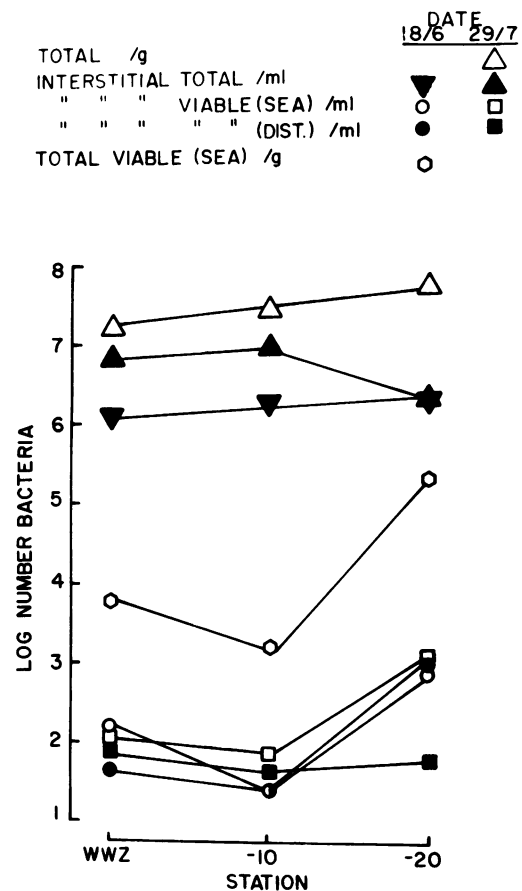


FIG. 1. Distribution of bacteria (viable and total, per gram of sand or milliliter of interstitial water) in Sindbad beach in the wave wash zone (WWZ) and two stations on a transect perpendicular to the wave wash zone. The -10 and -20 refer to 10 and 20 m inwards from the top of the WWZ. The standard deviations of counts on Millipore field monitors were 7 to 31%, whereas the standard deviations of counts on agar plates were between 22 and 39%.

TABLE 1. Comparison of viable counts per gram of sand on PYE-sea water medium and on PYE-distilled water medium from both the WWZ and WT during the spring and summer of 1971

Sample (Date) <sup>a</sup>	PYE-distilled water	PYE-sea water
WWZ (3/4)		$1.0 \times 10^3$
WT (3/4)		$1.4 \times 10^3$
WWZ (19/4) <sup>b</sup>		$1.4 \times 10^4$
WT (19/4)		$4.0 \times 10^4$
WWZ (15/7)	$1.6 \times 10^2$	$5.2 \times 10^3$
WT (15/7)	$5.3 \times 10^2$	$9.2 \times 10^3$
WWZ (22/7) <sup>c</sup>	$5.5 \times 10^2$	$5.2 \times 10^3$
WT (22/7)	$5.3 \times 10^2$	$3.4 \times 10^3$
WWZ (31/7)	$0.7 \times 10^2$	$7.8 \times 10^2$
WT (31/7)	$1.0 \times 10^2$	$8.3 \times 10^2$
WWZ (19/8) <sup>c</sup>	$7.8 \times 10^2$	$1.6 \times 10^3$
WT (19/8)	$2.7 \times 10^3$	$1.2 \times 10^3$

<sup>a</sup> (Day/month.)

<sup>b</sup> Immediately after a heavy storm; all other samples were taken after 1 or 2 weeks of calm.

<sup>c</sup> Average of two independent samples on 22/7 and eight independent samples on 19/8. For 19/8 the standard deviation for the WWZ was  $4 \times 10^2$  for PYE-distilled and  $8.1 \times 10^2$  for PYE-sea water; for the WT the standard deviations were  $1.7 \times 10^3$  for PYE-distilled and  $0.3 \times 10^3$  for PYE-sea water. In all, 41% of the independent samples fell outside of the standard deviation limits. The standard deviation for all samples on PYE-distilled was  $1.39 \times 10^3$  and for PYE-sea water was  $1.81 \times 10^3$ .

isolates. Rods were the major morphological type (88% of all isolates) and comprised 96% of the major group, group 3, the oxidative group (lacking the ability to ferment). The fact that oxidative organisms are the dominant group (59%) is consistent with the selective effect on aerobic plates and the presence of oxygen in interstitial water. Makemson (unpublished data) has shown that the percent saturation of oxygen was only once as low as 27% (1.41 ml of O<sub>2</sub>/liter), at 20 m inwards from the top of the WWZ. The normal values of oxygen saturation were between 50 to 75% in the WWZ as well as in the fresh water area in the water table (10 and 20 m inwards).

Although anaerobic pockets can exist in aerated soils (8), it seems that in the Sindbad beach the chance to develop such an anaerobic environment is rare, since the interstitial water is in constant flux. Rapid washing of the WWZ with sea water (15) combines with a substantial fresh water movement to the sea through the WT. The later causes a rather steep salinity gradient: 10 m inwards from the WWZ it was common to find a value of 18 to 30% salinity.

Thus, it is doubtful that anaerobic pockets could develop in the sand to make free-living anaerobic (fermentative) bacteria a dominant component of the total bacteria present. However, meiofauna and other metazoans in the beach may provide anaerobic environments in their gut cavities, thereby selecting and enriching for anaerobic or fermentative bacteria. This may account for the significant number of fermentative bacteria (41%) in our collection.

Out of the eight carbon sources tested to support the aerobic growth of the isolates as sole carbon sources, lactate appeared to be the more universal carbon source, which 43% of the isolates could utilize (Table 3). Although this study did not determine what carbon sources these isolates are utilizing in situ, these data show that a large percentage of the versatile fermenters have the ability to utilize lactate.

Although the number of sole carbon sources tested was not as extensive as the 146 single carbon sources studied in aerobic and fermenta-

TABLE 2. Physiological groups of bacteria isolated from Sindbad beach sand

Group	Physiological characteristics	Morphology	No. of isolates
1a	Ferments all four sugars <sup>a</sup>	Rods	57
b	Ferments all four sugars <sup>a</sup>	Cocci	7
2a	Ferments some, not all sugars <sup>a</sup>	Rods	74
b	Ferments some, not all sugars <sup>a</sup>	Cocci	11
3a	Oxidative (nonfermentative)	Rods	187
b	Oxidative (nonfermentative)	Cocci	26

<sup>a</sup> Glucose, lactose, sucrose, mannitol, as described in the text.

TABLE 3. Percent distribution of the ability to grow on individual carbon sources with ammonia as the sole nitrogen source

Carbon source	Group						Total
	1a (57) <sup>a</sup>	1b (7)	2a (74)	2b (11)	3a (187)	3b (26)	
Glucose	27	33	20	5	30	31	27
Galactose	22	33	14	5	27	23	23
Mannitol	24	17	24	5	29	12	25
Starch	17	17	17	5	13	23	15
Alanine	2	17	4	5	14	0	9
Aspartate	0	0	1	0	3	0	2
Acetamide	2	0	1	0	9	4	6
Lactate	70	67	22	5	46	31	43

<sup>a</sup> Number of isolates in each group. The groups are those specified in Table 2.

TABLE 4. Comparison of Sindbad Beach bacteria with bacteria isolated from open marine conditions<sup>a</sup>

Characteristic	Percent distribution among the isolates					
	Open marine bacteria			Beach bacteria		
	Aerobic (218) <sup>b</sup>	Fermentative (146)	Total (364)	Aerobic (213)	Fermentative (149)	Total (362)
Growth of single carbon sources						
Glucose	65.8	100	79.5	30.1	22.2	26.8
Galactose	33.3	44.5	37.8	26.5	17.6	22.8
Mannitol	36.1	82.9	54.9	26.9	22.3	25.0
Starch	ND <sup>c</sup>	ND	ND	14.2	16.4	15.0
L-Alanine	85.8	84.9	85.4	12.3	4.2	8.8
Aspartate	48.4	39.9	44.9	2.8	0.7	1.9
Acetamide	1.4	0	0.8	8.5	1.3	5.5
Lactate	89.5	90.6	89.9	44.1	41.6	43.1
Oxidase	87.2	95.8	90.6	87.3	49.0	71.6
Amylase	11.8	77.5	38.2	23.5	38.4	29.6
Nitrate reduction	16.5	94.5	47.8	8.0	6.0	7.2
Acetoin production	ND	16.5	ND	12.8	22.3	16.6
Indole production	ND	ND	0.07-1.5	52.7	59.7	55.5

<sup>a</sup>Data for the aerobic open marine bacteria from Bauman et al. (3) and for the fermentative open marine bacteria from Bauman et al. (4) are used in all cases except for the total percent indole production of open marine bacteria, which comes from Klug and DeMoss (10), in which case the total number of isolates does not apply.

<sup>b</sup>Number of isolates in each category. Aerobic are groups 3a and 3b, and fermentative are groups 1a, 1b, 2a, and 2b in Table 2.

<sup>c</sup>ND, Not determined.

TABLE 5. Percentage distribution of biochemical and growth characteristics among isolates in each physiological group

Characteristic	Physiological groups						Total
	1a	1b	2a	2b	3a	3b	
Acetoin production	28	15	22	0	13	12	16.6
DNase	78	57	52	67	54	85	60.5
Gelatin liquefaction	19	0	10	0	0.4	4	5.3
Gram positive	2	19	4	27	0	4	2.6
Gram negative	98	81	96	73	100	96	97.4
Indole production	63	86	58	5	52	58	55.5
Motility	0	0	100	0	99	0	71.6
Nitrate reduction	4	15	6	5	9	0	7.2
Oxidase	37	50	55	62	86	95	71.3
Starch hydrolysis	29	43	41	67	23	27	29.6
Growth on PYE-distilled water	34	33	34	23	25	27	28.5
Growth at:							
0 C	12	50	12	0	17	19	15.6
4 C	75	83	50	38	64	62	62.3
15 to 37	100	100	100	100	100	100	100
50 C	29	33	15	23	17	36	20.3
pH 4.0	23	0	8	10	3	0	7.1
pH 5.0	41	33	24	38	23	19	26.4
pH 6.0	95	100	74	85	82	82	82.9
pH 7 to 9	100	100	100	100	100	100	100

tive marine bacteria by Bauman et al. (3, 4), we compared their data with our isolates in Table 4. Marine bacteria isolated by Bauman et al. (3, 4) were, to a large extent, isolated from single carbon enrichments, and all of their isolates were isolated from the open Pacific Ocean. These open marine isolates appear to be more versatile than those from Sindbad beach. For example, 100% of the fermentative and 65.8% of the oxidative marine bacteria used glucose as a sole carbon source, compared to only 30% of the aerobic and 22% of the fermentative beach bacteria. Except for the utilization of acetamide, the open ocean marine bacteria were nutritionally more versatile than beach bacteria. Starch was not tested in the papers by Bauman et al. (3, 4). The presence of amylase was more prominent in the aerobic beach bacteria than those from the open sea. The reverse was the case when comparing the fermentative isolates. The apparently higher nutritional versatility of the offshore isolates compared to beach isolates may have been caused by the different modes of isolation.

Indole production has been demonstrated from 55 to 78% of bacteria isolated from marine invertebrate guts (10). In comparison, indole-positive organisms seem to be less prevalent in estuarine sediments (30-40%), in estuarine

water (14–18%), in open ocean inhabitants (4–5%), and in open ocean water (0.07–1.5%). Over half of the bacteria isolated from Sindbad beach were indole positive (Table 4). The trait was widespread among the physiological groups (Table 5). If it is correlated with invertebrate gut inhabitation, the data would suggest a symbiotic association of our isolates and the resident invertebrates.

The beach isolates were deoxyribonuclease positive, gram negative, motile, and oxidase positive. All had a growth preference for 15 to 37 C and pH 7 to 9. The motile organisms were all polarly flagellated. Only one aerobic isolate was peritrichously flagellated, and none of groups 1a, 1b, 2b, and 3b was motile. Only a few of the isolates liquefied gelatin, reduced nitrate to nitrite, or could grow below pH 5. Roughly 25 to 34% of the isolates could grow on PYE made with distilled water. This test was performed over a year after initial isolation on PYE-sea water media. From fresh sand, only 9.4% of the bacteria which grew on PYE-sea water agar also grew on PYE-distilled water media. The variation in these counts (excluding the August 19th sample) was remarkably consistent at both the WT and WWZ (3.1 to 15.6%). In the August 19th sample, the bacteria which grew on PYE-distilled water agar were 43.8% of those growing on sea water agar in the WWZ and 225% in the WT. Although the number of samples was limited, bacteria capable of growth on fresh water medium seem to be a minor component located on the fresh water side of the beach. The percent of bacteria growing on sea water agar averaged 70% of the total viable bacterial count in the WT and WWZ.

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