

The Relative Abundance and Seawater Requirements of Gram-Positive Bacteria in Near-Shore Tropical Marine Samples

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Received: 2 May 1994; Revised: 13 July 1994

Abstract. The relative abundance of gram-positive bacteria in a variety of near-shore marine samples was determined using the KOH method. Gram-positive bacteria accounted for 14%, 25%, 31%, and 12%, respectively, of the colony-forming bacteria obtained from seawater, sediments, and the surfaces of algae and invertebrates. A total of 481 gram-positive strains were isolated representing a wide range of morphological groups including regular and irregular rods, cocci, and actinomycetes. Seventy-seven percent of the strains characterized did not form spores and were aerobic, catalase-positive rods with regular to irregular cell morphologies. Eighty-two percent of the strains tested showed an obligate requirement of seawater for growth. None of the cocci tested required seawater or sodium for growth. This is the first report documenting that gram-positive bacteria can compose a large percentage of the culturable, heterotrophic bacteria associated with the surfaces of tropical marine algae.

Introduction

It was originally estimated that 95% of the bacteria in the sea are gram-negative [38]. Subsequently, low numbers of gram-positive bacteria have been reported from a variety of marine samples including seawater [1, 12, 25, 31], algae [8, 16, 18], plankton [29], invertebrates [10, 34], and the surfaces of fish [9, 11, 21] and submerged materials [7]. Higher numbers of gram-positive bacteria have been reported from sediments [4, 24, 28, 36], and this may be due to adequate nutrient availability [28]. Today, although it is generally accepted that culturable marine bacteria are predominately gram-negative, little quantitative data are available from which the percentage of gram-positive bacteria in marine samples can be calcu-

lated. The relative abundance of gram-positive bacteria in most marine habitats therefore remains unknown.

The lack of information describing gram-positive marine bacteria may be due to not only the relatively low numbers in which they have been reported from marine samples but uncertainties as to their origin and activity in the sea. These uncertainties are valid considering that gram-positive bacteria are abundant in soils and can be introduced into near-shore marine environments where their survival rates have not been determined. The extent to which gram-positive isolates can be considered indigenous marine bacteria can, at least in part, be assessed by their requirements of seawater and sodium for growth, two criteria traditionally used to distinguish marine from terrestrial bacteria [22, 39]. Unfortunately, with the exception of a few studies [e.g., 13, 14, 18, 26, 35], the seawater and/or sodium requirements of gram-positive marine isolates have not been reported.

We have observed that gram-positive bacteria can represent a large percentage of the colony-forming bacteria obtained from near-shore, tropical marine samples and that their relative abundance varies depending on the type of sample collected. In response to this observation, the following study was initiated to assess the relative abundance, seawater requirements, and morphological and physiological characteristics of gram-positive bacteria isolated from seawater, sediments, and the surfaces of marine algae and invertebrates collected off the coast of Belize, Central America.

Methods

Sample Collection

Field studies were conducted in June 1991 as part of a research expedition aboard the R/V *Columbus Iselin* (University of Miami). Samples were collected from 10 similar, well-developed, coral reef locations ranging in depth from 12 to 20 m off the coast of Belize. At each of the 10 locations, scuba divers collected two to four samples of seawater ($n = 32$), sediment ($n = 39$), algae ($n = 34$), and invertebrates ($n = 29$). Seawater was collected ~1 m above the sediment surface in empty, sterile, 50-ml polypropylene screw-cap tubes. Sediments, algae, and invertebrates were collected in similar tubes filled with sterile seawater.

The algae and invertebrates collected appeared healthy, and a given genus or species was collected only once at any location. The algae collected were *Lobophora variegata* ($n = 9$), *Rhizocephalus phoenix* ($n = 3$), *Styopodium zonale* ($n = 1$), *Halimeda* sp. ($n = 10$), *Microdictyon* sp. ($n = 3$), *Udotea* sp. ($n = 2$), *Dictyota* sp. ($n = 3$), *Dictyosphaera* sp. ($n = 2$), and unidentified alga ($n = 1$). The invertebrates collected were gorgonian soft corals and sponges. The gorgonians were *Gorgonia ventalina* ($n = 2$), *Briareum asbestinum* ($n = 7$), *Plexaura flexuosa* ($n = 1$), *Muriceopsis flavida* ($n = 2$), *Plexaurella nutans* ($n = 1$), unidentified gorgonian ($n = 1$). The sponges were *Agelas* sp. ($n = 2$), *Niphates digitalis* ($n = 3$), *Xestospongia* sp. ($n = 2$), *Callyspongia plicifera* ($n = 1$), *Iotrochata* sp. ($n = 2$), *Aplysina* sp. ($n = 2$), *Amphimedon compressa* ($n = 1$), and unidentified sponges ($n = 2$).

Bacterial Isolation, Gram Testing, and Characterization

Bacteria were isolated using standard serial dilution and plating techniques. All samples were processed aboard ship on the day of collection. Seawater samples were vortex mixed, and serial tenfold dilutions prepared to a final concentration of 10^{-6} with sterile seawater. For sediments, the overlying

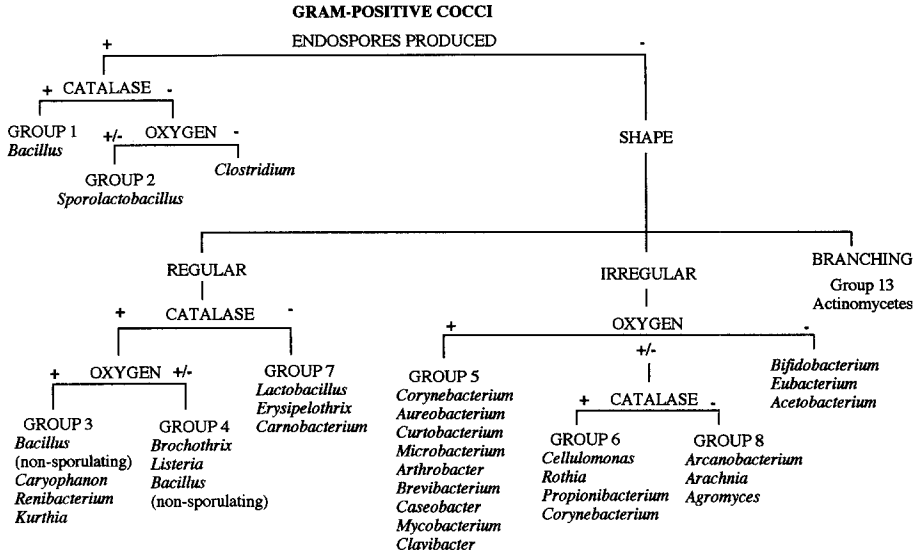


Fig. 1. Generic groups of gram-positive rods based on common morphological and physiological characteristics. For oxygen, (+) = aerobic, (+/-) = facultative anaerobe, (-) = anaerobe.

seawater was decanted, and the sediment was thoroughly mixed with a sterile spatula. Following mixing, 1 ml of wet sediment was volumetrically diluted as per the seawater samples. For algae and invertebrates, thin sections with 1 cm² surface areas were surgically removed, and the surfaces were gently scraped and rinsed with 10 ml of sterile seawater. The resulting solutions were diluted as per the seawater samples.

Diluted samples were inoculated onto medium I3 (2.5 g starch, 0.5 g yeast extract, 1.0 g peptone, 0.1 g sodium glycerol phosphate, 750 ml filtered seawater, 250 ml deionized water, 17 g agar, and filter-sterilized cyclohexamide added to a final concentration of 75 µg/ml after autoclaving to retard fungal contamination) and spread with a bent sterile glass rod. Inoculated plates were incubated at room temperatures (20–24°C). After 3–5 days, one plate yielding between 3 and 100 well-isolated colonies was selected to represent each sample. All bacterial colonies on the selected plates were gram tested using the KOH method [6] unless considered too small or overgrown by a neighboring colony. Even very small colonies could be identified as gram-negative using the KOH method. The selected plates were monitored for a total of 18 days, during which time at least two additional attempts were made to gram test all newly formed bacterial colonies. All colonies testing gram-positive or giving uncertain results were obtained in pure culture by repeated transfer onto fresh I3 plates and retested for Gram's reaction (KOH method).

Gram-positive bacteria were separated into 13 groups according to two schemes (Figs. 1, 2) prepared from material presented in references 2 and 33. Only genera that stain gram-positive were included in Figures 1 and 2. Anaerobes were not isolated in this study and therefore have not been included in the 13 groups, although they are listed in Figures 1 and 2. All strains were observed microscopically (1,000× magnification) on at least three separate occasions during various stages of growth. Bacteria were considered to be morphologically irregular if they showed a rod-to-coccus life cycle, rudimentary branching, or highly irregular filaments. Strains with well-developed branching hyphae were placed in the actinomycete group. Endospore formation was assessed by heat test [32] at 70°C for 10 minutes and microscopically in wet mount. Because the ability of bacteria to produce endospores can be difficult to demonstrate, the genus *Bacillus* (nonsporulating) was included in generic groups 3 and 4.

Table 1. Taxonomic assignments of gram-positive bacteria

Group ^a	Habitat				Total
	Seawater	Sediments	Algae	Invertebrates	
1. <i>Bacillus</i>	8	7	6	2	23
3. <i>Bacillus</i> (nonsporulating)	24	113	85	17	239
4. <i>Brochothrix</i>	15	10	8	1	34
5. <i>Corynebacterium</i>	14	37	27	7	85
6. <i>Cellulomonas</i>	3	1	0	2	6
8. <i>Arcanobacterium</i>	0	0	1	0	1
9. <i>Sporosarcina</i>	0	0	1	0	1
10. <i>Micrococcus</i>	3	3	1	1	8
11. <i>Staphylococcus</i>	2	3	9	0	14
12. <i>Streptococcus</i>	0	0	2	1	3
13. Actinomycetes	1	2	3	0	6
NA ^b	3	20	37	1	61

^aGenera listed are representative of each group; see Figs. 1 and 2 for a complete list of genera within each group.

^bNot available: Some strains were lost through contamination, became unculturable, or could not be assigned to a group by the methods employed here.

soft coral *Briareum asbestinum*, and gram-positive bacteria accounted for, on average, 9% of the bacteria tested ($n = 7$, $SD \pm 9\%$).

Four hundred and twenty (87%) of the gram-positive bacteria isolated in this study were assigned to generic groups (Figs. 1, 2) (Table 1). The results of these assignments indicate no apparent association between a generic group of bacteria and the type of sample collected (although these associations remain possible). Fifty-seven percent of all strains characterized did not form spores and were aerobic, catalase-positive rods with regular cell morphologies (group 3). Four genera are in this group, and none of the strains isolated appeared as multicellular rods with trichome formation as is characteristic of the genus *Caryophanon*. Bacteria belonging to group 5 were the second most abundant and can be distinguished from group 3 by having irregular cell morphologies. Irregular cell morphology has been reported for gram-positive marine bacteria [36]. However, the distinction between regular and irregular morphology can vary depending on growth phase and culture conditions. In total, 77% of the strains characterized in this study belonged to groups 3 and 5. No representatives of either group 2 (*Sporolactobacillus*) or group 7 (*Lactobacillus*) were isolated. It must be emphasized that Figures 1 and 2 are not derived from current evolutionary concepts of bacterial phylogeny; instead, they group together bacterial genera that share common morphological and physiological characteristics.

In total, 82% of the gram-positive bacteria tested required seawater for growth (Table 2). Approximately 90% of the strains from groups 3 through 6 required seawater. These four groups represent 87% of the bacteria characterized in this study. Although only 13 strains were tested for sodium requirements, there was a perfect correlation between the requirement of seawater and sodium for growth. The four strains of cocci tested grew in medium B1 prepared with sodium-free ASW and therefore either did not require sodium for growth or had low level

Table 2. Seawater requirements for the growth of gram-positive bacteria

Generic group ^a	Strains tested	Strains requiring seawater
1. <i>Bacillus</i>	18	11
3. <i>Bacillus</i> (nonsporulating)	161	147
4. <i>Brochothrix</i>	31	27
5. <i>Corynebacterium</i>	51	46
6. <i>Cellulomonas</i>	5	5
8. <i>Arcanobacterium</i>	1	0
9. <i>Sporosarcina</i>	1	0
10. <i>Micrococcus</i>	5	0
11. <i>Staphylococcus</i>	10	0
12. <i>Streptococcus</i>	1	0
13. Actinomycetes	6	1

^aGenera listed are representative of groups; see Figs. 1 and 2 for complete list of genera within each group.

sodium requirements (the concentration of sodium in sodium-free ASW was 0.27 mM). The final concentration of sodium in medium B1 prepared with sodium-free ASW was not determined but was higher than 0.27 mM because of the presence of sodium in yeast extract and peptone.

Discussion

The results presented here indicate that gram-positive bacteria can represent a large percentage of the colony-forming, heterotrophic bacteria associated with algal surfaces and sediments. The large relative abundance of gram-positive bacteria reported for algal surfaces can be attributed to their consistent association with two algal genera, *Lobophora* and *Halimeda*. To the best of our knowledge, this is the first observation of its kind reported for any algal species. Although the nature of this association remains unclear, it can be concluded that the distribution and relative abundance of gram-positive bacteria in specific marine habitats warrants additional study.

The large relative abundance of gram-positive bacteria associated with *Halimeda* and *Lobophora* is in striking contrast to studies of temperate marine algae, where virtually no gram-positive bacteria were reported [19, 20, 27]. One possible explanation for the associations reported here is the selective attraction of gram-positive bacteria to certain algal genera. Another possibility is that gram-positive bacteria adhere more effectively to the surfaces of some algae, or grow better on certain algal exudates, than do gram-negative strains. The attraction of specific bacterial taxa to algae, or their enrichment on algal surfaces, may help explain why previous studies have shown that algal-associated bacteria can be distinct from adjacent seawater communities [5, 15, 18, 20]. It would be interesting to determine if gram-positive bacteria isolated from the surfaces of *Halimeda* and *Lobophora* show a positive chemotactic response to the exudates or extracts of their respective algal host. If so, the phycosphere, as proposed by Bell and Mitchell for microalgae [3], may for some species of macroalgae be enriched with gram-positive bacteria.

Attempts to determine the gram reaction of all colony-forming bacteria using the KOH method [6] were successful as judged by the testing of 62% (algae) to 80% (seawater) of all visible colonies. To the best of our knowledge, this represents the first quantitative attempt to determine the relative abundance of colony-forming, gram-positive bacteria in marine samples. It must be emphasized that the results reported here refer only to colony-forming bacteria and therefore cannot be extrapolated to the entire bacterial community. Although it is generally accepted that colony-forming bacteria represent a small fraction of the total bacteria [e.g., 17, 30], this generalization does not appear to apply to all marine samples, because similar colony-forming and total bacterial counts have been reported for surface-associated bacteria [20]. Additional studies, possibly employing techniques in molecular biology, may help elucidate the true numbers of gram-positive bacteria in marine samples.

The KOH method was chosen for this study as a rapid and simple method to determine gram reaction. Although this method is comparable to gram staining [6], it can be misleading when used to establish phylogenetic relationships. The KOH method (as does gram staining) inherently assigns as gram-negative certain genera (e.g., *Megasphaera*, *Selenomonas*, *Sporomusa*) that, based on genetic analysis, belong to the gram-positive phylum [37]. The value of the gram reaction, therefore, is as a method to recognize bacteria that share a common cell wall structure. The observation that bacteria with gram-reaction-positive cell walls (which have long been neglected in studies of marine bacteria) can represent a large percentage of the culturable bacteria in certain marine samples sets the stage for future study of the ecological roles of these bacteria in marine systems.

More than 80% of the gram-positive bacteria tested in this study required seawater for growth. Thirteen strains were tested for sodium requirements, and those that required seawater also had a demonstrable sodium requirement. These bacteria can be defined as marine based on traditional proposals [22, 39]. Based on these same proposals, strains that do not require seawater or sodium for growth can be classified as halotolerant terrestrial bacteria. But should we make this assumption a priori and as a result eliminate non-seawater/sodium-requiring bacteria from discussions of marine bacteria? We believe that by restricting marine bacteria to include only those strains that meet a predefined set of requirements we run the risk of overlooking certain groups of bacteria that may be important in microbiological processes in the sea.

In support of this belief, it is interesting to note that none of the cocci tested in this study required seawater or sodium for growth. In at least two additional reports, gram-positive cocci isolated from marine samples did not require seawater for growth [18, 35]. Macleod [23] makes an interesting comment regarding the gram-positive coccus *Gaffkya homari* (name changed to *Aerococcus viridans*), the pathogenic bacterium that causes septicemia in lobsters. The author (citing unpublished data) states that *G. homari* does not require seawater for growth and that, at least among the gram-positive species in the sea, some representatives of the indigenous flora require neither seawater nor sodium for growth. Despite these observations, marine isolates that do not require seawater or sodium for growth are often described as terrestrial contaminants, without any attempt to assess their activities or ecological roles in the sea.

In conclusion, gram-positive bacteria composed 25% and 31%, respectively, of

the culturable, heterotrophic bacteria isolated from sediments and algal surfaces. Approximately 80% of the gram-positive strains characterized were aerobic, non-spore-forming rods with regular to irregular cell morphologies, suggesting that certain gram-positive taxa may predominate in the sea. Most of the bacteria tested required seawater for growth and therefore represent obligate marine bacteria. Cocci composed only 6% of the bacteria isolated, and none of those tested required seawater or sodium for growth. Based on their relative abundance, gram-positive bacteria have been shown to represent an important component of the culturable, tropical marine microbiota, and it remains possible that these bacteria have developed previously undocumented associations with certain species of marine algae.

Acknowledgements. We thank M.K. Harper, J. Wheaton, and J. Norris for invertebrate and algal identifications. We thank the Belizian government for permission to work in their territorial waters, P. Porcile and P. Zierer for technical assistance, and the crew of the R/V *Columbus Iselin* (University of Miami). We also thank Professors K. Neelson, B. Hemmingsen, and two anonymous reviewers for helpful critiques of this manuscript. This research is a result of financial support from the National Institutes of Health, National Cancer Institute, grants CA 50750 and CA44848, and the National Science Foundation (grant CHE90-08621).

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