Effect of Different Seawaters on the Development of Biochemically Deficient Mutants of *Serratia marinorubra*

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**Abstract**

CARLUCCI, A. F. (University of California, San Diego, La Jolla), AND S. B. SILBERNAGEL. Effect of different seawaters on the development of biochemically deficient mutants of *Serratia marinorubra*. Appl. Microbiol. 13:663-668, 1965.—The effect of different seawaters on survival and growth of biotin-, isoleucine-, and uracil-requiring mutants of the marine bacterium, *Serratia marinorubra*, has been investigated. Samples of seawater were collected from coastal waters, the California Current, and central North Pacific waters at depths of 1, 25, 50, 100, 250, and 500 m. The growth or survival of the test bacterium in basal medium prepared in these seawater samples was determined. The control water was synthetic or charcoal-treated natural seawater. In several experiments, the metabolite required by the bacterium was added to the basal medium 24 hr after inoculation, and the growth response was determined. Depending on the source, the seawater samples were both stimulating and inhibitory. Surface waters were more inhibitory than those taken at depth, where, in some cases, bacterial growth occurred. Seawater inhibition was related more to station depth than to the location of the station. The most toxic effects were found against the uracil-requiring mutant; the least, against the isoleucine-requiring mutant. The results of these studies and some laboratory experiments indicate that seawater toxicity is not primarily associated with the physical and biological properties of a particular water mass and that the same factor(s) may be responsible for the rapid death of bacteria in all waters.

Low bacterial populations of marine bacteria have been observed in the open sea (ZoBell, 1946; Kriss, 1963). The cell numbers vary with location and depth, the lowest counts being observed in deeper waters. Most of the data for the abundance of bacteria in the sea have been obtained from colony counts made with the use of highly enriched and selective media which support only a small fraction of the total population (Jannasch and Jones, 1959).

The minimal concentration of organic nutrients required for multiplication of most heterotrophs ranges from 10 to 100 mg per liter (ZoBell, 1946), although ZoBell and Grant (1943) and Jannasch (1958) reported that much lower concentrations of peptone or glucose could support growth of certain bacteria. Since oceanic seawater contains only about 1 mg of organic carbon per liter (Duursma, 1961), it may be considered a very dilute culture medium. Various organic compounds, both stimulating and inhibitory, in seawater have been reported (Sax et al., 1963; see reviews by Vallentyne, 1957; Saunders, 1957; Provasoli, 1963).

Jones (1963) concluded that numbers of bacteria in the ocean are limited by factors other than energy sources. He suggested that traces of toxic ions or a shift in the redox potential was responsible for the growth suppression of freshwater bacteria and a marine bacterium. Artificial seawater was used as the control water. More recently, Jones (1964) reported that heavy metals in seawater inhibit the growth but not the respiration of *Escherichia coli*. Chelating agents or metal-complexing substances overcome this toxicity of seawater.

A majority of the studies on inhibitory effects or bactericidal action of seawater have involved freshwater or sewage organisms, generally *E. coli*. The subject has been adequately reviewed (Pearson, 1956; Greenberg, 1956; Orlob, 1956; Carlucci and Pramer, 1959).

In bioassaying seawater with biochemically deficient mutants of a marine bacterium, *Serratia marinorubra*, requiring specific metabolites, seawater was found to suppress the growth of the mutants, and their response to added metabolites was masked by seawater toxicity. The present
Materials and Methods

Media. The composition of the solid enriched medium used was as follows: Casitone, 2 g; peptone, 2 g; yeast extract, 2 g; agar, 20 g (all Difco products); and aged natural seawater, 1 liter. The agar was omitted if enriched liquid medium was required. The composition of the basal liquid medium employed in this work, unless otherwise stated, was: glycerol, 10 ml; NH₄NO₃, 40 mg; sodium glycerophosphate, 20 mg; and seawater, 1 liter. In some experiments, 20 mg of K₂HPO₄ were used instead of sodium glycerophosphate. The seawater was either synthetic (Lyman and Fleming, 1940) or natural, and was supplemented with the constituents of the basal medium and sterilized by passage through a Millipore filter. Stock solutions of these supplements were sterilized separately by autoclaving at 15 psi for 15 min. Before inoculation with cells of the bacterium, this basal medium was precipitate-free and had a pH of 7.9. Solutions of the metabolites (biotin, isoleucine, and uracil) required by the mutants were sterilized by passage through Morton fritted-glass filters of ultrafine porosity, and were added aseptically.

Inoculum. Biochemically deficient mutants of S. marinorubra, requiring biotin, isoleucine, and uracil, were employed throughout these studies. The methods by which they were isolated and their descriptions were reported by Belser (1959). Cells grown on agar slants were suspended in sterile synthetic seawater and washed twice. After final centrifugation, the cells were transferred to synthetic seawater, and the suspension was adjusted to give a concentration of 10⁷ to 10⁸ cells per milliliter, as judged by visual estimates of turbidity. A 0.1-ml amount of a dilution of this suspension served as inoculum. The final concentration of cells in each basal medium prepared with the seawater sample was approximately 10⁸ cells per milliliter.

Experimental. In each experiment, a duplicate 10-ml sample of basal medium was prepared with the seawater under investigation. A control basal medium was prepared with charcoal-treated or synthetic seawater. Vessels were aluminum-capped tubes, 18 by 150 mm. Glassware was soaked in chromate cleaning solution, rinsed thoroughly in tap, deionized, and glass-distilled waters, and baked. After inoculation, each sample was incubated at 28 C in the dark. Platings on enriched medium to determine viable cells were made at intervals up to a maximum of 216 hr. A 0.1-ml amount of each sample or diluted sample was spread by a glass rod on the agar surface of each of three replica plates. Plates were incubated at 28 C, and colonies were counted after 48 hr.

Results

Experiments in enriched and basal media prepared with synthetic or natural seawaters. In a preliminary experiment, responses to specific metabolite in basal medium prepared in synthetic seawater by the biotin-, isoleucine-, and uracil-requiring mutants were investigated. During 120 hr of incubation, cell numbers of the biotin-requiring mutant growing in 100 μg of biotin per liter increased from 10⁴⁻¹ to 10⁴⁻², the isoleucine-requiring mutant in 1 mg of isoleucine per liter increased from 10³⁻⁴ to 10⁻¹, and the uracil-requiring mutant from 10⁻³ to 10⁻⁴ in 1 mg of uracil per liter. The inoculum level differed for each mutant, but a subsequent experiment (Fig. 1) showed that survival curves in the basal medium prepared in natural seawater by the uracil-requiring mutant were similar regardless of the initial cell concentrations.

The growth responses of the isoleucine-requiring mutant were determined in basal medium containing 1 mg of isoleucine per liter and in enriched liquid medium. The basal and enriched media were prepared with synthetic seawater. Figure 2 shows that the growth response of the mutant was poor in basal medium, favorable in basal medium containing 1 mg of isoleucine per liter, and best in enriched medium. The favorable effect of the constituents of the enriched medium may be due not only to their providing nutrients and growth factors but also to their chelating toxic ions in seawater (Jones, 1964).

The effect of synthetic and natural seawaters in...
FIG. 2. Development of isoleucine-requiring mutant of Serratia marinorubra in basal and enriched media prepared with synthetic seawater.

FIG. 3. Response of isoleucine-requiring mutant of Serratia marinorubra in basal medium prepared with natural and synthetic seawaters.

basal medium on the development of the isoleucine-requiring mutant was evaluated. Amounts of 0.1 and 1 mg of isoleucine per liter were included in each medium. Figure 3 shows that, after an initial lag in basal medium prepared in synthetic seawater, the isoleucine-requiring mutant gave a similar growth rate to that obtained in basal medium made in natural seawater; both media contained 1 mg of metabolite per liter. A 0.1-mg amount of isoleucine per liter was favor-}

able for growth only in natural seawater. If metabolite was not added, the population died most rapidly in synthetic seawater.

Since natural seawater was passed through a PH Millipore filter before use in basal medium preparation, the possibility of cell rupture and subsequent release of metabolite or inhibitor from the indigenous population was investigated. Natural seawater was passed through a PH Millipore filter, and the filtrate was used to prepare basal medium. Synthetic seawater was next passed through the same filter, and the filtrate was used to prepare basal medium also. The control basal medium was made in synthetic seawater which was passed through a new filter on which no particulate organic matter was present. Before inoculation, all basal media were allowed to equilibrate (with pH and dissolved gases) at room temperature overnight. Figure 4 shows that with each mutant growth or survival was better in the synthetic seawater which was passed through the filter containing the natural seawater residues. To minimize cell rupture, the vacuum in the filtration unit was kept at 2 psi.

Experiments in basal medium prepared in seawater collected in North Pacific Ocean. Seawater samples were collected in cleaned Frautchy bottles on California Cooperative Fisheries Investigations Cruise 6301–2 in January, 1963. Depths were 1, 25, 50, 100, 250, and 500 m in all stations except Station 52 along Line 60 in a southwest direction from San Francisco (Fig. 5).
Depths did not exceed 75 m in Station 52. Stations 52 and 60 were in coastal waters; 80, 90, and 100 were in California Current waters; and 160, 180, and 200 were in central North Pacific waters.

The degrees of shading of boxes in Fig. 6, 7, and 8 show the effects of different seawaters on the three mutants when specific metabolites were not added. The shading of the box appearing for each sample was determined by calculation of the slope of the survival curve obtained after 216 hr of incubation.

The biotin-requiring mutant was inhibited by all waters except those collected below a depth of 25 m in Station 60 and in five of the nine samples collected at depths greater than 50 m in Stations 80, 90, and 100. Conditions favorable for survival were observed in waters collected from 250 m in Station 160, from 250 and 500 m in Station 180, and from 50 and 100 m in Station 200. Waters collected at 250 m in Stations 90 and 160 were very stimulating to the mutant.

The most toxic waters were in the shallower depths from Stations 80, 160, 180, and 200. In all stations, surface waters were inhibitory to the bacterium. The stimulating or inhibitory effects of seawaters on the biotin-requiring mutant appeared to be associated more with depth than with the stations from which they were collected.

Seawaters, in general, were less inhibitory to the isoleucine-requiring mutant than to the biotin-requiring mutant (Fig. 7). In two samples, surface waters from Stations 52 and 80, the isoleucine-requiring mutant died rapidly.

Consideration of waters from all depths in the California Current and central North Pacific revealed little difference in the number of samples from each water mass which were stimulating to the bacterium. Waters from different depths in each water mass, however, varied in their stimulating effects on the bacterium.

The uracil-requiring mutant was inhibited more than the biotin- and isoleucine-requiring mutants (Fig. 8). The bacterium did not grow in any of the waters tested. All the waters from coastal Station 52 were strongly inhibitory, and most of the samples from 100 m and above in the central North Pacific were also strongly inhibitory.

In another series of experiments, the growth responses of the biotin- and uracil-requiring mutants were evaluated in basal media prepared with the seawater samples and supplemented with specific metabolites. Seawater samples from depths of 100, 250, and 500 m in all stations were used to prepare the basal media. Each solution was inoculated with the test mutant and, after...
from the wild type by ultraviolet light irradiation grow in basal medium if sufficient metabolite is added.

An unfavorable growth response of the particular mutant to seawater may be due not only to the presence of toxic substances but also to the lack of specific or related metabolite. The mutants tend to die in basal medium prepared with charcoal-treated or synthetic seawater when specific metabolite is not added. A bacterial death rate greater than that obtained in such water indicates the presence in natural seawater of toxic substances. Conversely, a reduced death rate indicates that the seawater in question is more beneficial to the bacterium.

The results of the present studies have shown that the effect of seawater on *S. marinorubra* varies from mutant to mutant and is influenced by the location and depth from which seawater samples are taken. In survival studies by means of experiments in which external metabolites were not added, surface waters were found to be most inhibitory. Jones (1963) found that wild-type *S. marinorubra* was stimulated by surface waters but that growth was suppressed by samples taken at 25, 50, and 250 m from coastal waters 10 to 12 miles west of San Diego. However, the media he used were more enriched than those employed in the present studies.

Jones (1964) found that *E. coli* grew in seawater only after a prolonged lag phase and that the beneficial effects of various added organic materials were manifested in a reduced lag phase. In our experiments, in which specific metabolite was added during the lag phase 24 hr after inoculation, some of the waters showed stimulatory effects but others showed no effect when compared with unsupplemented controls.

Saz et al. (1963) reported that the anti-*Staphylococcus* factor in seawater is a large nondialyzable, heat-labile molecule and is responsible, in part, for preponderance of gram-negative organisms in the sea. In some of our experiments (not reported above), the seawater used to prepare the basal media was charcoal-treated, but survival of the biotin-requiring mutant in this water was not different from its survival in untreated seawater.

Therefore, it seems unlikely that the toxic effect of seawater in this case was due to the presence of large organic molecules (Johnston, 1955; Jones, 1959; Saz et al., 1963; Provasoli, 1963). The types of organic materials reported to be present in seawater, which may be inhibitory or stimulating, include carbohydrates (citric, formic, glycolic, lactic, and malic acids), fatty acids (up to 20 carbons), amino acids (free or from hydrolysates), and vitamins. The forms in which the above

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**Fig. 8.** Maxima slopes (those which predominated for at least 120 hr) over a 216-hr incubation time of the uracil-requiring mutant in basal medium prepared with seawaters obtained from various locations and depths.

**Table 1.** Development of biotin- and uracil-requiring mutants in basal medium prepared in seawater collected from various locations and supplemented with 1 mg of specific metabolite per liter 24 hr after inoculation*

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<thead>
<tr>
<th>Depth</th>
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* I. Exponential growth by biotin-requiring mutant; no growth by uracil-requiring mutant. II. Exponential growth by uracil-requiring mutant; no growth by biotin-requiring mutant. III. Exponential growth by biotin- and uracil-requiring mutant. IV. Exponential growth by biotin-requiring mutant; slight growth by uracil-requiring mutant. V. Slight growth by uracil-requiring mutant; no growth by biotin-requiring mutant. VI. No growth by either mutant.

24 hr of incubation at 28 C, 1 mg of specific metabolite per liter was added. Incubation was continued to 216 hr, and viable cell counts were determined periodically. Table 1 summarizes the results of six experiments.

**Discussion**

Wild-type *S. marinorubra* is a rapidly growing gram-negative rod (Belser, 1959). It develops well in a basal medium containing glycerol as the sole source of energy, and it can tolerate a wide range of salinities. The biotin-, isoleucine- and uracil-requiring mutants which have been obtained

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identified compounds exist in seawater is questionable because of the isolation procedures used. Since biotin- and isoleucine-requiring mutants grew in some of the waters studied (the growth curves had positive slopes), it is reasonable to assume that these samples contained the specific metabolite or related compounds which could be utilized by the bacteria.

Although the problem of inhibitory or stimulating effects of different seawaters on bacteria is complex, three general observations may be made. Waters from all stations and depths were inhibitory to the uracil-requiring mutant, deep samples from coastal and central North Pacific waters were favorable for the biotin-requiring mutant, and samples from waters at all depths were more or less favorable for the isoleucine-requiring mutant. Since inhibition occurred to some extent in all water masses at all depths, it seems unlikely that inhibition is caused by substances characteristic of any of the water masses. However, some factor seems to be present in most surface waters which inhibits the growth of the three mutants. At other depths the situation is more complex, but no definite pattern emerges which is obviously related to the physical or biological oceanography of the regions that have been studied.

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LITERATURE CITED


