Evidence that *Escherichia coli* Accumulates Glycine Betaine from Marine Sediments

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*Escherichia coli* grew faster in autoclaved marine sediment than in seawater alone. When *E. coli* was cultivated in sediment diluted with minimal medium M63 at 0.6 M NaCl, supplemented or not supplemented with glucose or with seawater, the osmoprotector glycine betaine was accumulated in the cells. The best growth occurred on glucose. Accumulation of glycine betaine was not observed when *E. coli* was grown in sterile seawater alone. The fact that *E. coli* grew better in the sediments than in seawater is attributed somewhat to the high content of organic matter in the sediment but mainly to the accumulation of glycine betaine. Thus, osmoprotection should be considered to be an additional factor in bacterial survival in estuarine sediments.

From the viewpoint of public health, the presence of enteric bacteria in seawater is of great concern. Survival of enteric pathogens in estuarine and marine environments has been the subject of extensive study for nearly a century. It has been assumed that many chemical, physical, and biological factors lead to the decline and disappearance of pathogenic bacteria in seawater (5, 6, 22). Among these factors, dilution, sedimentation, and salinity are considered important. However, during recent years, new bacteriological techniques allowing better detection of these bacteria have provided evidence that allochthonous bacteria, as well as autochthonous bacteria, are capable of entering a viable but nonculturable state (14, 18, 24, 31). Stressed cells survive for long periods in seawater. The gram-negative bacteria probably enter into dormancy, during which they remain viable and potentially virulent (9). Furthermore, several workers (13, 16, 25, 27) demonstrated a longer survival time of *Escherichia coli* in estuarine sediments than in seawater that is attributed to the greater content of organic matter in the sediment than in seawater.

On the other hand, it is well established that osmoprotective substances, mainly betaines, found in plants (29), algae (3, 4), marine invertebrates (2), and vertebrates (32) were involved in protecting cells against high salt concentrations. Glycine betaine (G-B) is accumulated by *E. coli* and other members of the *Enterobacteriaceae* from growth media and restores growth in high-salt-content media (20, 21). Thus, when betaines are released into the marine environment because of death of the organisms, excretion, or terrestrial supply, they become available as osmoprotectors for bacteria.

In the present study, we confirm the observations of Gerba and McLeod (13), that the better survival of *E. coli* in the presence of sediments than in seawater was dependent on organic matter. Furthermore, we prove that, in vitro, *E. coli* accumulates G-B from autoclaved estuarine sediments; this would explain the better survival in sediments in the presence of high salt concentrations. These marine sediments supply not only a carbon source but also osmoprotection. Consequently, osmoprotector accumulation by bacteria constitutes another step in studies of the survival of enteric pathogenic bacteria in marine and estuarine environments.

**MATERIALS AND METHODS**

**Collection of water and sediment samples.** Sediment samples, consisting of mud from mud flats, were collected at the estuary of Morlaix Bay (Brittany, Northwestern France), which receives effluents from a sewage treatment plant and runoff from upstream rural communities. Seawater samples were collected in sterile glass bottles. Salinity (34 to 35%) was determined with a Beckman type RS7C salinometer. All samples were kept cool in an ice chest until they were transported to the laboratory, where they were kept at 4°C until use. All samples were used within 24 to 48 h of collection.

**Organisms.** *E. coli* ZB400, ZB402, and ZB403 were isolated from the marine environment by direct plating onto Drigalski's medium (Institut Pasteur Production, Manne La Coquette, France). Identification was confirmed by Gram stain morphology, the ability to ferment lactose with gas production at 44°C, and a negative oxidase reaction and by using the API 20E diagnostic system (API System S.A., Montalieu Vercieu, France).

**Inoculum preparation.** The bacteria were incubated aerobically in 5 ml of Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth (AES Laboratoire, Combourg, France) at 37°C for 18 h. They were then washed three times with 5 ml of saline solution (0.9% [wt/vol] NaCl in distilled water). One milliliter of a washed cell suspension (about 109 cells ml−1) was used to inoculate 1 liter of medium.

**Growth in sediment.** Sediment (10% [wt/vol]) was mixed with minimal medium M63 (8) at 0.6 M NaCl, supplemented or not supplemented with 10 mM glucose, or with seawater to produce a total volume of 1 liter. Seawater supplemented with 100 mM KCl, 15 mM (NH4)2SO4, 0.16 mM MgSO4·7H2O, 3.9 μM FeSO4, and 10 mM glucose was used as the control. Media were sterilized at 110°C for 30 min. They were then inoculated with bacteria and incubated at 37°C for 24 h with agitation.

**Bacterial enumeration.** Total viable culturable bacteria were enumerated by spread plating onto Trypticase soy agar. All culture plates were incubated for 24 h at 37°C.

**Extraction of intracellular betaine.** Cultures were decanted
overnight at 4°C to eliminate solid sediment. Several 750-ml portions of supernatant containing bacterial cells were centrifuged at 3,000 × g, and the pellets were washed twice with M63 containing 0.68 M NaCl. The washed cells were then extracted with 10 ml of 70% (vol/vol) ethanol at room temperature for 20 min with occasional gentle agitation (20). The cells were sedimented by centrifugation, the supernatant was collected, and the extraction process was repeated on the pellets three times with 70% (vol/vol) ethanol. The ethanol extract was evaporated to dryness, redissolved in 5 ml of distilled water, lyophilized, and redissolved in 500 µl of distilled water before analysis. Sediments (10 g) were extracted with 70% (vol/vol) ethanol (100 ml) and treated in the same way.

Betaine purification. To isolate the osmoprotective compound, the crude extract obtained was subjected to high-voltage electrophoresis for 75 min at 40 V · cm⁻¹ using Whatman 3MM paper previously moistened with 0.75 N formic acid (pH 2.0). After being dried in an aerated oven (40°C), the electrophoretograms were sprayed with Dragendorff reagent to detect quaternary ammonium or tertiary sulfonium compounds. The strips of paper recovered without reagent and containing the compound accumulated by E. coli were cut into small pieces and extracted with 70% (vol/vol) ethanol. The extract was vacuum dried, dissolved in distilled water, and chromatographed by using an 80% phenol–ethanol mixture (1:1) as the solvent. After being dried, the chromatograms were sprayed with Dragendorff reagent. Finally, the compound was eluted in 70% (vol/vol) ethanol, the solvent was evaporated, and the extract was redissolved in distilled water and lyophilized. After this, the extract was considered purified. In all cases, G·B and choline (Sigma Chemical Co., St. Louis, Mo.) were used as standards.

Identification by thin-layer chromatography. The extracts were chromatographed on silica gel 60-Kieselgel F₂₅₄ thin-layer chromatography plates (thickness, 0.2 mm) (E. Merck AG, Darmstadt, Federal Republic of Germany) by using methanol-NH₄OH (3:1) or isopropanol-H₂O (3:1) as the solvent (11). The plates were exposed to I₂ vapor or were sprayed with Dragendorff reagent. In this last case, colors produced were intensified by overspraying with a saturated solution of sodium hydrogen sulfate (3). G·B, sarcosine, trigonelline, carnitine (Sigma), γ-butyrobetaine (Aldrich Chemical Co., Strasbourg, France), and dimethylsulfonio-proponate, as described previously (7), were used as standards.

¹H-NMR spectroscopy and mass spectrometry. ¹H-nuclear magnetic resonance (¹H-NMR) spectroscopy was conducted on a Bruker AM 300WB spectrometer operating in a Fourier transform mode at 300.134 MHz. The samples were dissolved in D₂O. D₂O also provided the signal for the NMR field lock. Sodium 3-(trimethylsilyl) propane sulfonate was added as an internal standard. Mass spectrometry was carried out on a Varian Mat 311 spectrometer coupled with gas chromatography with the following parameters: electron energy, 70 eV; accelerating voltage, 3,000 V; temperature of direct-insertion probe, 180°C. Spectrum was in electronic impact.

Quantitative determination. Quantitative determination of -onium compounds (quaternary ammonium and tertiary sulfonium) (30) was done by the method of Wall et al. (28) as modified by Storey and Wyn Jones (26). Sediment samples (10 g each) were extracted by agitation (i) in 100 ml of 70% (vol/vol) ethanol, centrifuged, evaporated to dryness, and dissolved in 5 ml of distilled water; (ii) in 100 ml of M63 at 0.6 M NaCl added with glucose and sterilized at 110°C for 30 min; or (iii) in 100 ml of distilled water at room temperature. In all cases, 125-µl samples were used for analysis.

Biological assay of the purified substance. Before the unidentified compound accumulated by cells was subjected to different analytical operations, it was experimented upon to determine its effectiveness in bacterial osmoregulation. After recovery of the purified lyophilized substance, a portion was dissolved in distilled water and used for biological assay. Ten-, 50-, and 65-µl portions of the solution were added to 5 ml of M63 at 0.85 M NaCl supplemented with glucose to determine the osmoprotective compound uptake rate. Then they were inoculated with 5 µl of a washed suspension of E. coli ZB400 and incubated with shaking at 37°C for 48 h. Growth was assessed by plate counting.

RESULTS

E. coli growth. In the presence of sediments, the growth yield of E. coli ZB400, ZB402, and ZB403 reached a maximum at 24 h and then stabilized at about 9 × 10⁶ CFU/ml. An identical profile was observed for all three strains (Table 1). On the other hand, no growth was observed in seawater—cell numbers remained constant (about 3 × 10⁶ CFU/ml). Surviving strains were maintained at the same level throughout the experiment (Table 2). Since the maximal growth observed on sediments was at about 24 h (Table 1), cultures grown for G·B purification were used at this time. Enumeration results (Table 3) show that the best growth of E. coli strains was obtained when sediments were diluted with M63 at 0.6 M NaCl supplemented with glucose (10⁸ CFU/ml). When sediments were diluted with seawater or M63 at 0.6 M NaCl, the cell numbers were about 10⁷ CFU/ml. Finally, seawater alone allowed no growth; however, bacterial survival was observed in seawater (about 10⁶ CFU/ml).

Osmoprotective effect of the purified substance. Results concerning the osmoprotective role of the still unidentified compound are illustrated in Fig. 1. When E. coli ZB400 was cultivated in M63 at 0.85 M NaCl, inhibition of growth was caused by the high osmolality of the medium. When 10 µl of the compound was added, growth was restored. Growth was improved by the addition of 50 or 65 µl of the unidentified compound.

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<th>Time (day)</th>
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M NaCl added with glucose and sterilized at 110°C for 30 min; or (iii) in 100 ml of distilled water at room temperature. In all cases, 125-µl samples were used for analysis.

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Seawater was supplemented with 100 mM KCl, 15 mM (NH₄)₂SO₄, 0.16 mM MgSO₄·7H₂O, 3.9 µM FeSO₄, and 10 mM glucose.
G·B identification. From all cultures obtained from sediments and with all separation techniques used, the unknown substance accumulated by E. coli was co-electrophoresed and co-chromatographed with pure G·B. In the first case, the Rf (migration distance of G·B divided by the migration distance of choline) was 0.31. In the second case, the Rf was 0.90. Migration of the purified substance obtained by thin-layer chromatography provided the same Rf. Rf were 0.68 and 0.34, respectively, when methanol-NH4OH (3:1) or isopropanol-water (3:1) was used as the solvent system.

No G·B accumulation was detected when cell extracts were obtained from seawater culture in the absence of sediments. However, when sediment was present, G·B was detected rapidly in all extracts, especially when glucose was added to the sediment. For this reason, all the extracts were gathered and subjected to 1H-NMR spectroscopy and mass spectrometry identification.

It must be pointed out that a second Dragendorff-positive compound with an Rf of 0.70 was inconstantly and weakly revealed by electrophoresis. This means that E. coli strains accumulated this compound in very small amounts. On the other hand, G·B was detected in ethanolic extracts of cells from raw sediments, but detection required more time.

1H-NMR spectroscopy and mass spectrometry. 1H-NMR spectroscopy was previously used for G·B determination in E. coli (19). The 1H-NMR spectrum showed that the chemical shifts of the two peaks were at 3.27 and 4.03 ppm and coincided exactly with the shifts of a standard G·B.

The mass spectrum showed the free base (CH3)2-N-CH2-COOH, but the molecular ion was not observed. The theoretical mass for C4H9NO2 was 103.0633, and the mass found was 103.0634.

Betaine dosage. Under the conditions of our experiment, -onium compounds were not detected when sediments were diluted with M63 at 0.6 M NaCl that was supplemented with glucose and autoclaved or in distilled water. The ethanolic extract of cells from sediments contained 0.6 μmol of -onium compounds per ml.

DISCUSSION

Salinity is considered to be the essential characteristic of seawater. Also, it constitutes one factor in the decline of enteric bacteria in seawater (1, 6). Many researchers (12, 14, 15, 31) have demonstrated the survival of enteric pathogenic bacteria in marine environments. Furthermore, this survival is better in sediments (15, 24, 25, 27), where bacteria are concentrated. Sedimentation is considered to be one factor in the disappearance of bacteria from seawater. However, to our knowledge, no information has been presented concerning the ability of E. coli or other bacteria to survive osmotic stress caused by the high salinity of seawater. Osmoprotective substances, mainly betaines, are known for their role in restoring the growth of Enterobacteriaceae (20, 21, 23) and other species of bacteria (for a review, see reference 10). Our results are evidence that E. coli cells growing on autoclaved marine sediments resist salinity stress by accumulating and concentrating G·B from sediments.

As reported by Gerba and McLried (13), nutrients bound to the sediments are easily released by autoclaving, and higher concentrations of organics in the sediment than in the water constitute a contributing factor in the longer survival of E. coli in the sediment. Autoclaving facilitates breakdown of macromolecules, availability of the macromolecules, and consequently better nutrient assimilation. This was confirmed when glucose was used as an additional carbon source. In this case, the growth yield reached 106 CFU/ml, whereas in the absence of glucose, growth of the three E. coli strains on sediments reached only about 104 CFU/ml after 24 h. Even when supplemented with 10 mM glucose, seawater allowed no growth. Only survival was observed (106 CFU/ml), which was probably the result of the lack of nutrients. Thus, the quantity and the nature of organic matter present in seawater constitute predominant factors that favor the growth and maintenance of enteric pathogenic bacteria in marine environments.

Furthermore, E. coli strains used in this study accumulated G·B from marine sediments regardless of the salted dilution medium used. No accumulation was observed when E. coli was grown in seawater alone. Thus, sediments constituted the only G·B source. Since the best growth was observed on sediments, when glucose was used as an additional carbon source, the highest accumulation of osmoprotective compound occurred. On the other hand, the small amount of G·B detected in sediments proves that E. coli cells accumulated G·B in order to alleviate osmotic stress caused by salt. Another Dragendorff-positive compound (probably another betaine), when detectable, was accumulated by E. coli cells but in very small amounts. The fact that G·B, a powerful osmoprotector, was accumulated in non-negligible quantities from sediments demonstrated that G·B
constitutes an important osmoprotective source in marine environments and that it seems to exist in larger amounts than other osmoprotectors. The osmoprotective compound G - B is widespread in plants as well as in marine animals and algae (2, 4, 29, 32). Thus, not surprisingly, it is recovered from the marine environment. As reported by King (17), the G - B content is quite high within the upper layer of sediments associated with an algal mat in a hypersaline pond. However, G - B is diluted in seawater and relatively concentrated in salt-laden sediments.

Summary. This study confirmed that autoclaved marine sediments constituted an important organic matter source and protected E. coli cells from osmotic stress in seawater by supplying G - B. However, these results must be confirmed by in situ studies. It is essential to know whether G - B (or other osmoprotectors) is easily released from sediments and concentrated by cells since it was reported that nutrients bound to the sediment material were not easily released into the nonautoclaved natural seawater (13).

Survival of enteric pathogens in marine environments is of great interest in the context of public health. In addition of organic material present in seawater, osmoprotectors such as G - B should contribute to the maintenance of pathogenic bacteria and explain the survival of cells in salted environments.

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LITERATURE CITED