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Characteristics of *Escherichia coli* Grown in Bay Water as Compared with Rich Medium†

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Membrane-filtered bay water can support a certain degree of growth of *Escherichia coli* organisms isolated from the bay water or from sewage. The effect of the growth medium (bay water versus rich medium) on sensitivities to antimicrobial agents and cell envelope proteins was studied in many of these strains. Bay water-grown cells were less sensitive to bacteriophages and colicins, but were more sensitive to heavy metals and detergents as compared with rich-medium-grown cells. These results indicated that the cell envelope composition of the bay water-grown cells could be modified, resulting in altered susceptibility to various antimicrobial agents. An analysis of cell envelope proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that cells from rich-medium-grown cultures contained two or three major outer membrane proteins, whereas in bay water-grown cells, the OmpF protein was greatly reduced.

Water, which covers nearly three-fourths of the earth's surface, is the habitat of biochemically versatile microflora. Water bacteria play an indispensable role in nutrient cycles and in chemical and ecological processes. In studies of these organisms, laboratory media have been commonly used for cultivation. As a result, some data obtained from cultures grown in rich laboratory media may not represent the intrinsic properties of the aquatic organisms grown in their natural habitat. Bay water, which contains minute amounts of required nutrients, can support a limited degree of bacterial growth. In the Chesapeake Bay, bay water contains, per liter: 10 to 90 μg-atoms of N, including NO_3^-, NH_4^+, urea N, and NO_2^-; 0.6 to 1.8 mg-atoms of P; and about 15 mg of dissolved organic carbon (26, 35, 37). Therefore, cultivation in bay water can be used as a model system for the investigation of in situ properties of water bacteria. Unfortunately, no studies on the characterization of bacteria grown on natural bay water have been reported.

Escherichia coli, although not an indigenous water bacterium, was selected for use in this study for several reasons. *E. coli* is an important bacterium of public health concern and has long been used as an indicator organism for water quality and food safety (19, 20). This organism is well characterized in its genetics, biochemistry, physiology, and ecology. Thus, results from the present study can be readily compared with existing information. Furthermore, investigations of this organism grown in bay water may serve as a model for the study of opportunistic growth of bacteria and for the study of the mechanism of survival or adaptation of terrestrial organisms in an aquatic environment.

The cell envelope of gram-negative bacteria consists of three layers: the inner membrane, peptidoglycan, and the outer membrane. The cell envelope contains proteins, phospholipids, and lipopolysaccharide. The outer membrane of *E. coli* K-12 grown in laboratory media contains a few predominant proteins, including porin proteins OmpF and OmpC, heat-modifiable OmpA protein, and lipoprotein (3, 13, 31, 32). These major proteins are interspersed in the outer membrane bilayer with other cell envelope components to form a matrix-like structure which maintains the cell integrity and determines the cell permeability of nutrients and susceptibility to attack by external agents. Porins form channels in the outer membrane which allow nonspecific and passive transmembrane diffusion of small molecules such as sugars, amino acids, ions, and nucleotides (29, 30). These proteins also serve as receptors for bacteriophages and colicins (4, 7–10, 15). OmpA protein is postulated to play an important role in bacterial conjugation and also functions as a surface receptor for bacteriophages and colicins (6, 18, 25, 30). OmpA protein together with lipoprotein is necessary for maintaining the in-

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tegrity of cell outer membrane and cell morphology (24, 34); mutants lacking both proteins are extremely fragile and grow very slowly (33).

The composition of the cell envelope can be altered quantitatively and qualitatively when the cells are exposed to different growth conditions and environmental stresses (23). These changes in the cell envelope components may affect cellular physiology and cell survival, which is particularly important in the natural aqueous ecosystem. In this report I characterize the cell sensitivity to bacteriophages, colicins, detergents, and heavy metals together with the protein composition of cell envelope of bay water-isolated *E. coli* grown in filtered bay water, and I compare them with those of the same strains grown in rich laboratory medium.

MATERIALS AND METHODS

Bacterial strains and culture media. Test strains of *E. coli* (Table 1) were isolated from bay waters and from raw sewage from a local city (Crisfield, Md.) according to standard procedures (1, 36). Bay water strains of *E. coli* were isolated from the Chesapeake Bay, Mobile Bay, and Narragansett Bay. After confirmation tests, the identification was completed by IMViC tests (indole, methyl red, Voges-Proskauer, and citrate tests) and Gram staining (1, 36).

Difco Proteose Peptone beef extract and 0.5% NaCl was used as rich medium (PPBE) (6). Bay water from the Little Annemessex River, a tributary of the Chesapeake Bay, near Crisfield's Marine Products Laboratory, and from the Choptank River, near the Horn Point Environmental Laboratories, University of Maryland, with a salinity ranging from 10 to 13‰ was taken at high tide during the cold weather between late fall and early spring. The bay water was filtered immediately, first through three layers of Whatman no. 1 filter paper, then through a 0.45-μm membrane filter, and finally through a 0.20-μm membrane filter. The membrane-filtered bay water, after being tested for sterility, was stored at −20°C for later use. For preparation of bay water agar plates, a threefold strength of agar in bay water was prepared and autoclaved. One volume of this threefold strength of agar was melted, added to two volumes of membrane-filtered bay water, and poured into the plates. For the control agar plate, distilled water was used instead of bay water to prepare a distilled-water agar plate.

Growth of *E. coli* on bay water. Test cultures were grown in PPBE broth overnight at 35°C. The cells were washed three times with saline and finally suspended in the membrane-filtered bay water to an absorbance of about 0.05 at 650 nm. They were incubated at 35°C on a rotary shaker. For the control, cells were treated as described above except that buffered saline was used instead of bay water.

Isolation of colicins. The colicins of mitomycin C-induced cells of the respective colicinogenic strains were prepared by NaCl (1 M) extraction and precipitated with ammonium sulfate according to the procedure previously described (12). The colicin titers were determined as arbitrary units which were defined as the highest dilution giving a clear zone on the lawn of the sensitive host cells. The selected colicins used in this study are described in Table 2.

Preparation of bacteriophages. Bacteriophage stocks were prepared by lysis of host cells in broth at an early logarithmic growth phase (7). The bacteriophages used and their characteristics are described in Table 3.

Sensitivity to colicins, bacteriophages, heavy metals, and other chemical agents. The final colicin preparations were diluted in 20 mM phosphate buffer to a test concentration of 10 arbitrary units. Phages specific for outer membrane proteins, and other phages, were used at a final titer of 10^6 PFU/ml measured on a sensitive wild-type *E. coli* K-12 strain, JF568 or JF694 (7). Heavy metals and other chemical agents were

TABLE 1. *E. coli* strains used

Isolates	Isolation: Source	Isolation: Salinity (‰)	IMViC tests[a]	Reference
1–6	Chesapeake Bay, Little Annemessex River	13–14	+ + − −	This paper
7–10	Chesapeake Bay, Breton Bay	5–12	+ + − −	This paper
11	Chesapeake Bay, Patuxent River	11	− + − −	This paper
12–21	Chesapeake Bay, Easton Bay	4–13	+ + − −	This paper
22–28	Mobile Bay	5–25	+ + − −	This paper
29–31	Mobile Bay	5–25	− + − −	This paper
32–44	Narragansett Bay	1–28	+ + − −	This paper
45–56	Crisfield city sewage		− + − −	This paper
57–64	Crisfield city sewage		+ + − −	This paper
K-12 JF568 *aroA357 ilv-277 metB65 his-53 purE41 proC24 cyc-1 xyl-14 lacY29 rpsL77 tsx63*				7

[a] See text.

TABLE 2. Colicins used

Colicin	Mode of action	Reference
A	Blocks energized uptake of amino acids and sugars and, subsequently, inhibition of DNA, RNA, and protein synthesis; requires OmpF protein for reaction	8, 10
B	Affects energy metabolism; mode action similar to that of colicin A	10
E1	Disrupts energized transport and oxidative phosphorylation; inhibits synthesis of DNA, RNA, and proteins	18
E2	Inhibits DNA metabolism	10
E3	Cleavage of 16S RNA of 30S ribosomal units and, consequently, inhibition of protein synthesis	18
L	Inhibition of macromolecular synthesis, active transport, and reduction in cellular levels of ATP; requires OmpA protein for reaction	12

dissolved in distilled water or appropriate solvents at the given concentrations.

Test strains grown in bay water were compared with the same strains grown in PPBE broth for their sensitivities to colicins, bacteriophages, detergents, and heavy metals on the appropriate agar plates. Volumes of 0.25 ml of these antimicrobial agents at the test concentrations were placed in the wells of micro tissue culture plates. Then, they were transferred with a 48-tipped multireplicator to both bay water agar plates and PPBE agar plates on which the test cultures had already been seeded. To obtain a visible lawn, excess volumes (0.4 to 0.5 ml) of bay water-grown cells were spread on bay water agar and allowed to dry before antimicrobial agents were applied. Each spot on the agar test plate received about 0.015 ml of antimicrobial agent at the test concentrations. After incubation at 35°C overnight, the plates were examined for sensitivity. The formation of a clear spot or a cloudy zone on the lawn of the test strain was recorded as a sensitive reaction. The lack of inhibition of growth was recorded as resistance.

Preparation of cell envelopes. The cells were grown in 500 ml of PPBE in a 2-liter flask and incubated at 35°C on a rotary shaker to an absorbance at 650 nm of about 1.0. Cells were harvested by centrifugation at 4°C and washed with cold saline solution. The cell envelopes were prepared as previously described (6).

To prepare the cell envelopes of organisms grown in bay water, each tested strain in PPBE broth, at a late-logarithmic growth phase, was diluted 200-fold into 500 ml of filtered bay water in 2-liter flasks. The cells were harvested after incubation at 35°C for about three generations on a rotary shaker. Cell envelope material was prepared as described above (6). The protein content of cell envelopes was determined by the method of Lowry et al. (21), using bovine serum albumin as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Total cell envelope materials were solubilized by heating the samples at 100°C in sodium dodecyl sulfate buffer for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a dual slab gel cell (280 mm) was carried out in a discontinuous buffer system described by Lugtenberg et al. (22). Electrophoresis was terminated when the tracking dye electrophoresed to the bottom of the gel. Disk gel electrophoresis was performed in the same system and terminated 1 h after the tracking dye had electrophoresed off the gel. Gels were then removed, stained with 0.05% Coomassie blue in 10% acetic acid for 6 h, and destained in 10% acetic acid at 37°C overnight.

RESULTS AND DISCUSSION

***E. coli* isolates.** All *E. coli* isolates used in this study are listed in Table 1. A total of 44 *E. coli* cultures were isolated from the surface water of the Chesapeake Bay (21 strains), Mobile Bay (10 strains), and Narragansett Bay (13 strains) at different locations with salinities ranging from 1 to 20‰. Another 20 *E. coli* cultures were isolated from four independent sewage samples taken from the raw sewage of the city of Crisfield, Md. These organisms were gram negative and produced a typically metallic sheen on eosin methylene blue agar. They produced gas at 35°C in lauryl tryptose broth and brilliant green lactose bile broth, and at 44.5°C in EC broth. All except 1 of the 21 strains isolated from the Chesapeake Bay were typical *E. coli* (type I) with an IMViC pattern of + + − −. The one exeptional strain was atypical *E. coli* (type II) which had an IMViC pattern of − + − −. However, 60% (12 of 20) of city sewage isolates were atypical *E. coli* (type II). Only typical *E. coli* isolates were subjected to further characterization of cell envelopes.

Growth on bay water. Filtered natural bay water could support the growth of all freshly isolated bay water *E. coli* tested, although there

TABLE 3. Bacteriophages used

Bacteriophage	Receptor components	Reference
K3	OmpA	7
TuII*	OmpA and LPS[a]	9
TuIa	OmpF and LPS	9
T2	OmpF and LPS	15
TuIb	OmpC and LPS	9, 38
434	OmpC and LPS	15
T4	OmpC and LPS	28
T5	*tonA*-coded outer membrane protein	4
T6	*tsx*-coded outer membrane protein	14
T7	LPS	16
TC45	Protein E	7

[a] LPS, Lipopolysaccharide.

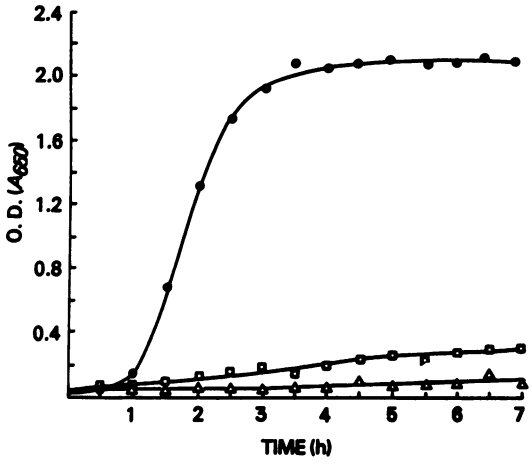

FIG. 1. Growth curves of bay water-isolated *E. coli* strain 13 incubated at 35°C in different media. Symbols: ●, rich medium (PPBE); □, bay water plus 0.1% glucose; △, bay water.

were variations in the degree of growth. Figure 1 shows an example of growth curves on both bay water and rich medium (PPBE) of a representative bay water *E. coli* isolate. Based on optical density and viable plate count, the generation time at an incubation temperature of 35°C was determined to be 190 min when grown on bay water as compared with 34 min on the rich medium at the same temperature. Bay water alone, without other nutrient supplements, could support the growth of about three generations, reaching a cell density of approximately 3×10^7 cells per ml. Longer incubation at 35°C tended to result in the lysis of some cells. In the control experiment, no detectable growth was observed when test cells were incubated in buffered saline. The addition of an amino acid mixture (leucine, methionine, proline, tryptophan, histidine, isoleucine, valine, and arginine) at 25 μg/ml into bay water did not increase the growth of the cultures tested (data not shown), nor was the growth rate increased when 0.05% ammonium chloride was added. When supplemented with 0.1% glucose, bay water supported growth with a generation time of about 100 min, reaching a final cell density of about 2×10^8 cells per ml. The results indicated that the bay water used was more deficient in a glucose C source than in an N source as regards the growth requirements of *E. coli*.

No growth was observed on distilled-water agar plates. This indicates that the nutrient residues on agar did not support the growth of test cultures. Earlier studies had demonstrated that heterotrophic bacterial populations are capable of growth in river water only over a narrow range of dilution rate of 0.005 to 0.027/h (17). They also showed that a portion of the total bacterial populations is absorbed onto the surface. In the present study I did not examine the fraction of bacteria absorbed by the glass surface.

Bacteriophage and colicin sensitivities. One of the intriguing aspects of the bay water-grown cells is their unusual resistance to bacteriophages and colicins. Both bacteriophages and colicins require certain cell surface receptors for a positive reaction. The sensitivity of a culture to these antimicrobial agents can show whether the tested culture contains the required cell surface component. All except 1 of the 44 bay water *E. coli* strains tested were resistant to many phages specific for outer membrane protein and to other common coliphages (Table 4). The single exception was sensitive only to phage TuIb. However, over 85% of these same organisms grown in rich medium were sensitive to one or more of these phages. A similar result was obtained from the fresh sewage isolates (data not shown). Of eight sewage isolates tested, one to eight strains were sensitive to any one of the bacteriophages tested when the cultures were grown on PPBE. When they were grown on bay water, the same cultures became resistant to all of these bacteriophages except T4 and T6. These results indicate that *E. coli* grown in the natural aquatic environment could be more resistant than has been expected. It also indicates that the cell envelope composition of these organisms might be modified when grown in the bay water, resulting in the loss or masking of bacteriophage receptors.

A similar observation was made with regard to colicin sensitivity (Table 5). Six colicins, A, B, E1, E2, E3, and L, were tested at a concentration of 10 arbitrary units; about 40% of the 44 strains of bay water *E. coli* grown in rich medium were sensitive to one or more of these

TABLE 4. Sensitivity to bacteriophages of *E. coli* cells grown on PPBE agar or bay water agar

Phage	PPBE agar		Bay water agar	
	No. of strains sensitive	No. of strains resistant	No. of strains sensitive	No. of strains resistant
K3	18	26	0	44
TuII*	4	40	0	44
TuIa	7	37	0	44
T2	8	36	0	44
TuIb	18	26	1	43
434	3	41	0	44
T4	17	27	0	44
T5	3	41	0	44
T6	11	33	0	44
T7	3	41	0	44
TC45	0	44	0	44

TABLE 5. Colicin sensitivity of bay water *E. coli* isolates grown on PPBE agar or bay water agar

Colicin (10 AU)[a]	PPBE agar		Bay water agar	
	No. of strains sensitive	No. of strains resistant	No. of strains sensitive	No. of strains resistant
A	4	40	0	44
B	3	41	0	44
E1	3	41	0	44
E2	9	35	0	44
E3	6	38	0	44
L	11	33	1	43

[a] AU, Arbitrary units (see text).

colicins. However, only one of these organisms, when grown in bay water, was sensitive to one of these colicins. Similar differences in colicin sensitivity (data not shown) were found between bay water- and rich-medium-grown cells of eight *E. coli* strains isolated from raw city sewage. Each of these eight sewage cultures (100%) was sensitive to 65% of the colicins for PPBE-grown cells, whereas only three of the eight cultures (37%) were sensitive to just one of the six colicins (17%) for bay water-grown cells.

The higher resistance of bay water-grown cells than PPBE-grown cells to bacteriophages and colicins has a significant implication for the survival of these organisms in the natural aqueous environment. These organisms grown in the natural bay water appear to have a higher resistance to bacteriocins and bacteriophages than those grown in rich medium under laboratory growth conditions. There are at least five possible explanations for the higher resistance of bay water-grown cells to various phages specific for outer membrane protein and to colicins: (i) cell envelope components or structure (or both) in the bay water-grown cells can be altered, resulting in the loss of phage and colicin receptors; (ii) the repulsion of phage particles or colicin molecules by ionic charges or electrostatic forces from the cell surface of bay water-grown cells may result in the failure of phage or colicin adsorption to the cells; (iii) the required cofactors for phage adsorption (2) or colicin action are present in rich medium but absent in bay water; (iv) excessive DNases may be present in the periplasmic space of bay water-grown cells, and the digestion of phage DNA during injection into the cells causes the loss of infectivity; and (v) bay water-grown cells may produce some exocellular structures to mask the phage and colicin receptors.

Sensitivity to heavy metals. In sharp contrast to bacteriophage and colicin sensitivity, bay water-grown cells were more sensitive than PPBE-grown cells to heavy metals, including $CuSO_4$, $HgCl_2$, $AgNO_3$, and $CoCl_2$ (Table 6). These results indicate that *E. coli* strains in the natural bay water environment could be more sensitive to heavy metals than strains grown on conventional rich laboratory media. Similar results were obtained with the sewage isolates, where bay water-grown cells demonstrated a higher sensitivity to heavy metals than PPBE-grown cells (Chai, unpublished data).

The possible causes for a higher sensitivity to heavy metals in bay water-grown cells than in PPBE-grown cells are as follows: (i) a reduction in the effective metal concentration, e.g., complex formation between the metal ions and the chelating agents present in PPBE-grown cells; or (ii) an increased intracellular concentration of the metals in the bay water-grown cells. The presence of a porin with a higher permeability of metal ions could be responsible for a more efficient transport of heavy metals into the cells. Alternatively, a high ionic charge could exist on the cell surface of the bay water-grown cells. The heavy metals present in the medium would exchange with the cell surface ions, resulting in a concentration of heavy metals higher than that in the medium.

Sensitivity to detergents and other chemicals. The sensitivity of *E. coli* to detergents, dye, and zwittergent is shown in Table 7. As with the heavy metals, bay water-grown cells were more sensitive to sodium dodecyl sulfate, Triton X-100, sodium sarcosinate, Zwittergent 3-12, and methylene blue than were PPBE-grown cells

TABLE 6. Sensitivity to heavy metals of *E. coli* cells grown on PPBE agar or bay water agar

Metal	Concn (%)	PPBE agar		Bay water agar	
		No. of strains sensitive[a]	No. of strains resistant[b]	No. of strains sensitive	No. of strains resistant
$CuSO_4$	0.2	4	40	44	0
$HgCl_2$	0.01	1	43	44	0
$Pb(Ac)_2$[c]	0.2	1	43	41	3
$AgNO_3$	0.01	23	21	44	0
$CoCl_2$	0.04	1	43	27	17

[a] A clear spot formed where the chemical agent was applied on the lawn of the tested strain.
[b] No inhibition of growth was found.
[c] Ac, Acetate.

TABLE 7. Sensitivity to detergents and other chemicals of bay water *E. coli* grown on PPBE agar or bay water agar

Chemical	PPBE agar		Bay water agar	
	No. of strains sensitive	No. of strains resistant	No. of strains sensitive	No. of strains resistant
SDS[a] (1%)	1	43	17	27
Triton X-100 (12%)	2	42	19	25
Sodium sarcosinate (1%)	0	44	18	26
Methylene blue (0.5%)	11	33	21	23
Zwittergent (1%)	0	44	7	37

[a] SDS, Sodium dodecyl sulfate.

under identical test conditions. These results indicate that cells grown in bay water might be more sensitive to these antimicrobial agents or more readily accessible to penetration by these antimicrobial agents. The changes in the sensitivity of bay water-grown cells to these antimicrobial agents again may reflect the modification of chemical composition of cell envelopes. Two possible explanations of why bay water-grown cells are more sensitive to detergents than PPBE-grown cells are: (i) cell envelopes of bay water-grown cells might bind a larger quantity of detergents than do those of PPBE-grown cells; and (ii) detergents might associate with bay water-grown cells with a higher affinity or a higher penetrability than with PPBE-grown cells.

Cell envelope composition. Because the different sensitivities to antimicrobial agents of PPBE- and bay water-grown cells might be due to alterations in cell envelope composition under different growth conditions, it is critical to determine whether differences in the major components of cell envelopes from the two types of cells do exist. Cell envelopes of 44 bay water isolates (Table 1), prepared both from bay water-grown cells and from rich-medium-grown cells, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). The results show that the cell envelopes of these organisms grown on PPBE contained two or three major outer membrane proteins. All strains tested contained a heat-modifiable protein that had an electrophoretic mobility like that of OmpA protein. The next most common major outer membrane protein was a peptidoglycan-associated protein with an apparent molecular weight of about 39,000 and an electrophoretic mobility similar to that of OmpC protein. A major outer membrane protein similar to OmpF protein was found in some of the *E. coli* organisms isolated from bay water. One major outer membrane protein with an apparent molecular weight of higher than 40,000 was found in some of the organisms tested, but was not present in the laboratory *E. coli* K-12 and B strains.

Cell envelopes prepared from bay water-grown cells showed that some *E. coli* strains have outer membrane protein patterns different from those of PPBE-grown cells, although a few strains showed no difference (data not shown). OmpF-like proteins and other 40,000-molecular-weight proteins disappeared or were greatly

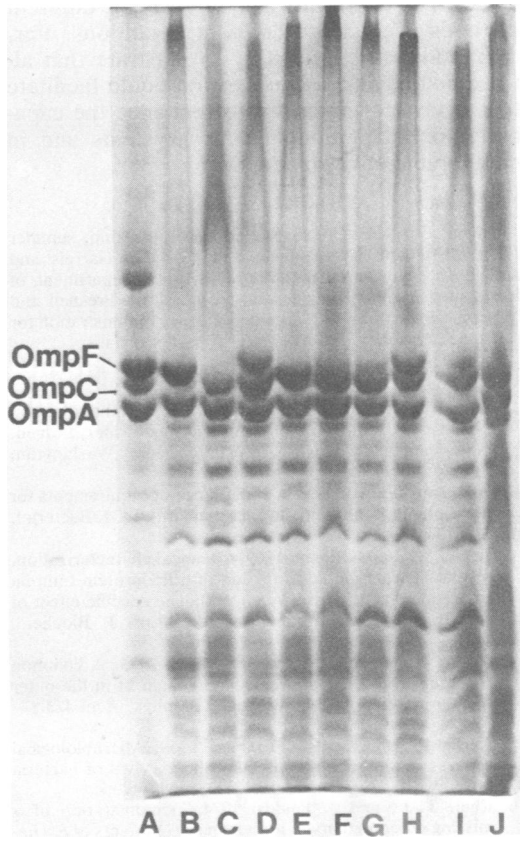

FIG. 2. Stained sodium dodecyl sulfate-polyacrylamide gels after electrophoresis of cell envelope materials prepared from PPBE-grown cells of strain K-12 JF568 (A) and bay water isolates 7 (B), 8 (C), 9 (D), 10 (E), 11 (F), 16 (G), 21 (H), 12 (I), and 13 (J).

reduced. However, the heat-modifiable proteins, OmpA protein or its related proteins, were not markedly altered by the growth in bay water.

The changes in cell envelope composition reflect the stress of growth in bay water as compared with that in rich laboratory medium. The altered cell envelopes, changed in protein composition, might modify the nutrient uptake efficiency of the cells and enhance the capacity of survival of bay water bacteria. This alteration in cell envelope composition might be beneficial to or necessary for the survival of the cells.

This study demonstrated that under the experimental conditions used, bay water could support a weak growth of *E. coli*. Some reports have demonstrated that *E. coli* organisms gradually die off in the natural aquatic environment (11, 27). Factors which cause the decrease in bacteria in natural water or seawater include sunlight, toxic substances, deficient nutrients, unfavorable temperature, predators, and competitive and antagonistic effects of other organisms (5, 17). One explanation of the discrepancy between my results and others is the different methods used under different conditions. Further studies are necessary to illustrate that altered cell envelope composition could facilitate the survival of organisms or enhance the capability for adaptation in water systems and in unfavorable environments.

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