Revista de Biología Marina y Oceanografía 33 (2): 325-330, diciembre de 1998

Characterization of *Escherichia coli* strains selectively isolated from seawater

Caracterización de cepas de *Escherichia coli* aisladas selectivamente de agua de mar

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ABSTRACT

Escherichia coli strains were enriched from coastal waters impacted by sewage discharges. Isolates were characterized in terms of growth response as a function of salinity and low temperature; in addition , patterns of resistance to antimicrobial agents and transmissibility of resistance markers were examined. We studied resistance to mercuric chloride, nalidixic acid, tetracycline, chloramphenicol, kanamycin, streptomycin and ampicillin.More than 50% of the strains showed salt tolerance and ability to grow at 7 °C in contrast with control *E. coli* strains. About 15% displayed resistance to one or more antimicrobial agents and 6% transferred resistance markers in genetic crosses including recipients of the genus *Vibrio*. Results suggest that the *E. coli* strains examined have an increased potential to persist in the coastal marine enviroment and might display genetic activity therein.

Key words: *Escherichia coli*, low temperature, salinity, genetic transmission, marine environment.

RESUMEN

Se aislaron cepas de *Escherichia coli* de aguas costeras impactadas por descargas de aguas servidas. Las cepas aisladas se caracterizaron por su respuesta de crecimiento en función de salinidad y baja temperatura; además, se examinaron sus patrones de resistencia a agentes antimicrobianos y la transmisibilidad de los marcadores de resistencia. Estudiamos resistencia a cloruro de mercurio, ácido nalidíxico, tetraciclina, cloranfenicol, kanamicina, estreptomicina y ampicilina. Más del 50% de las cepas eran tolerantes a la sal y crecían a 7 °C en contraste con cepas control de *E. coli*. Cerca de un 15% exhibían resistencia a uno o más agentes antimicrobianos y 6% transferían marcadores de resistencia en cruces genéticos incluyendo receptores del género *Vibrio*. Los resultados sugieren que las cepas de *E. coli* examinadas tienen un potencial incrementado para persistir en el ambiente marino costero en donde podrían exhibir actividad genética.

Palabras clave: *Escherichia coli*, baja temperatura, salinidad, transmisión genética, ambiente marino.

INTRODUCTION

Continual sewage discharges into coastal waters are of wide spread ocurrence and lead to the accumulation of fecal bacteria, such as *Escherichia coli*, which are known to survive and persist in the marine environment (Grimes *et al.* 1988, Gauthier *et al.* 1992) a situation that is probably enhanced by osmoprotective agents either in sediments or produced by algae (Ghoul *et al.* 1995).

The presence of *E. coli* as a contaminant of the coastal marine environment is well documented and the pioneering survey studies cited below indicate that the *E. coli* strains examined often contain transmissible genes coding for antibiotic resistance (Smith 1971). Regarding our coastal environment, Campos *et al.* (1987) evaluated fecal pollution in the bay of Valparaiso. Likewise, Silva *et al.* (1984, 1987) isolated strains of *E. coli* and other enterobacteriaceae from polluted seawater of the Antofagasta and Concepción bay areas. These authors also report the incidence of antibiotic resistance markers and their transmissibility to an *E. coli* K 12 receptor.

However, these reported results refer to *E. coli* strains isolated by standard methodology. No attempts have been made to selectively enrich for strains with attributes that could be indicative of an increased fitness in regard to the marine environment. Furthermore, transmission of antibiotic resistance markers to endogenous marine bacteria was not tested. Within this context our aims were to enrich *E. coli* strains using a seawater based medium and to characterize these bacteria in terms of their growth response to salinity and low temperature. Furthermore we sought to determine among them the incidence of (i) transmissible antimicrobial resistance markers and (ii) efficient genetic donors of such markers, particulary in crosses with recipients of the genus *Vibrio*.

MATERIALS AND METHODS

MEDIA AND SUPPLEMENTS

All bacteriological media and media components were from Difco (Detroit, Mi., U.S.A.). Antibiotics were from Sigma (St. Louis, Mo., U.S.A.) and salts from Merck (Darmstadt, Germany).

SELECTIVE ISOLATION OF *E. COLI* FROM SEAWATER

E. coli strains were isolated from seashore seawater samples collected in sterile tubes at sites nearby sewage discharge outlets, in the bay of Valparaíso, Chile. Samples were refrigerated (8-10 °C) and processed within 2h after collection. To enrich selectively for enterobacteria, 0.5 ml of sample was inoculated into a flask containing 50 ml of sterile medium made in seawater and containing 0.1% Tryptone and 0.05% yeast extract. After incubation at 40 °C for 24 h a loop of culture was streaked on Mac-Conkey Agar to obtain isolated colonies. One lactose positive colony from each enrichment was further purified on L agar which contains in g/l: tryptone, 10; yeast extract, 5; NaCl. 5; agar, 15.

STRAIN CHARACTERIZATION

E. coli strains were identified using the API 20 E system (Analytab Products, Plainview, N.Y.). Salt tolerance was assessed by ability to grow on L agar made in different dilutions of a 30% stock solution of a mixture of marine salts as described by Quesada *et al.* (1984). This solution contains the major ions in seawater: sodium, magnesium, calcium and potassium.

Fresh L broth cultures of *E. coli* isolates were spotted on L agar plates containing different salt concentrations and bacterial growth was recorded after incubation for 5 days at both 28 °C and 37 °C. Growth response at 7 °C was recorded after 12 days of incubation in L broth, starting with an inoculum of about 2 x 10^5 cells/ml , unless indicated otherwise.

Resistance to antibiotics and mercury was determined by growth after incubation at 37 °C for 24 h on L agar plates supplemented with different antimicrobial agents used in the following concentrations (in µg/ml): Hg Cl_2 (12.5 and 27); nalidixic acid (Nal; 50); tetracycline (Tc; 25); chloramphenicol (Cm; 20); kanamycin (Km; 50); streptomycin (Sm; 25) and ampicillin (Ap; 50). These concentrations are recommended for testing plasmid mediated resistance (Provence & Curtiss 1994)

GENETIC CROSSES

These were performed on the basis of methods described by Provence & Curtiss (1994) . Matings between drug resistant strains and genetic recipients were as follows: equal volumes of exponentially growing cultures of donors and recipients (1:1 ratio) were mixed in 1.5 ml Eppendorf tubes. Cells were pelleted by centrifugation for 30 sec. in an Eppendorf 5414 microcentrifuge at room temperature. After incubation under several

conditions (see Results) pellets were suspended by vortexing and 50 µl of the cell suspensions were then plated on appropriate selective media to recover recombinants. In each experiment separate cultures of donor and recipient were included as controls.

RESULTS

Using the seawater based medium enrichment procedure described in materials and methods we obtained positive enrichment cultures in all polluted seawater samples tested. Because the sample volume was only 0.5 ml it is likely that the bacteria recovered represent the most abundant clones persisting in our marine environment. Under these conditions, *E. coli* strains were preferentially selected; however, we also recovered a strain of *Klebsiella pneumoniae* that grows as efficiently as *Vibrio harveyi* in a seawater based medium (data not shown).

We finally examined a total of 47, independently isolated *E. coli* strains identified as such by the API 20E identification system. These strains were characterized in terms of salinity tolerance in comparison with a control group of *E. coli* strains isolated from nosocomial infections. Results indicating an incresed salt tolerance of the *E. coli* strains selectively isolated from seawater are in Table 1. In addition, over 50% our *E. coli* isolates also display the ability to grow at 7 °C (Table 2) in sharp contrast with the *E. coli* nosocomial isolates. We have further confirmed this inherent property of the seawater isolates by using lower inocula, of about 1 x 10^3 cells, obtaining the same results.

In terms of resistance to antimicrobial agents, only 8 out of the 47 strains examined showed single or multiple resistance (Table 3). However, only 3 out of 8 resistant strains examined transmitted resistance markers in genetic crosses with an *E. coli* recipient. All these strains donated their resistance markers "en bloc" with the exception of the Sm marker of *E. coli* strain 016, which was not transferable.

Table 1. Growth response towards salinity of *E. coli* strains enriched from seawater.

Tabla 1. Respuesta de crecimiento frente a salinidad de cepas de *E. coli* enriquecidas de agua de mar.

Salinity (%)	Strains showing growth (%)			
	Group A		Group B	
	28 °C	37 °C	28 °C	37 °C
0.5	100	100	100	100
3.0	100	100	100	100
5.0	100	100	100	100
7.5	100	100	94.7	84.2
10.0	29.8	70.2	0	0

[a] Group A: 47 strains of *E. coli* isolated from polluted seawater samples.

Group B: 19 *E. coli* nosocomial isolates kindly provided by Dr. P. Nercelles, Hospital Van Buren, Valparaíso.

Table 2. Growth response of *E. coli* strains incubated at 7 °C.

Tabla 2. Respuesta de crecimiento de cepas de *E. coli* incubadas a 7 °C.

N° tested[a]	Origin	Special features	Strains that grow (%)[b]
26	seawater	Drug sensitive	53.8
8	seawater	Drug resistant	62.5
19	nosocomial	Not determined	0

[a] Only 34 strains of the original 47 *E. coli* seawater isolates were examined due to loss of strains during storage.

[b] Growth response was recorded as positive when cultures reached turbidities equivalent to a concentration of $1x10^8$ cells/ml. A laboratory strain of of *E. coli* K-12 was also tested as negative control.

As shown in Table 3, the only strain that transmits its resistance markers at a higher frequency is *E. coli* 022. Moreover we have detected in strain 022 a single plasmid band that also appears in transconjugants that become Ap and Sm resistant (data not shown). We have named this plasmid as pUCV5 and have tested its transfer in genetics crosses performed under a variety of conditions. Results of these experiments are summarized in Table 4.

Table 3. Patterns of resistance to antimicrobial agents of *E. coli* seawater isolates.

Tabla 3. Patrones de resistencia a agentes antimicrobianos en aislados de *E. coli* de agua de mar.

Strain designation	Resistances						Transmission[a]
01		Sm					-
07			Tc	Cm	Km	Hg	-
015	Ap	Sm	Tc				-
016		Sm	Tc	Cm	Km	Hg	+
019		Sm	Tc	Cm	Km		-
022	Ap	Sm					++
026							N.D.
042	Ap	Sm					+
Frequency	37%	35%	75%	50%	37%	37%	

[a] (-) No transmission.
(+) Transmission at low frequency (about $1x10^{-3}$ transconjugants/donor cell or less).
(++) Transmission at higher frequency (about $1x10^{-3}$ transconjugants/donor cell or more).
N.D. Not determined. In all crosses the recipient was *E. coli* C600.

Table 4. Transmission of pUCV5 in heterologous crosses.

Tabla 4. Transmisión de pUCV5 en cruces heterólogos.

Donor[a]	Recipient[b]	Mating conditions[c]	Transmission factor[d]
E. coli 022	*Vibrio harveyi*	28 °C; 3h; L.N.	2
	VAL 505	As above	n.d.
	VAL 511	As above	n.d.
E. coli x1849 (pUCV5)	*V. anguillarum*	28 °C; 24h; M.A	2-3
	V. damsela	As above	2-3
	V. fluvialis	As above	n.d.
	V. ordalii	As above	n.d.
	V. vulnificus	As above	2-3

[a] For a description of *E. coli* 022 see text and Table 3. The *E. coli* x1849 (pUCV5) derivative is a multiauxotroph requiring diaminopimelic acid (DAP) for growth. Selection against this donor was performed by plating in media without DAP. *E.coli* x1849 was originally obtained from Dr. Roy Curtiss III.

[b] The *V. harveyi* recipients were Nal-resistant. Strain VAL 505 and VAL 511 were isolated from intestinal contents of *Venus* sp. and *Mytilus edulis*, respectively and rifampicin-resistant derivatives were used as recipients. *Vibrio* strains were obtained from Dr. Ana Baya, University of Maryland.

[c] M.A. is a marine medium prepared in 75% seawater and containig 0.5% bactopeptone and 0.1% yeast extract. In L.N. nutrients are 100-fold less concentrated than in M.A.

[d] Transconjugants of marine bacteria were recovered on M. A. selective plates. Numbers indicate the order of magnitude below the optimum transmission determined with a control *E. coli* recipient at 28 °C.

n.d. Transmission not detected.

DISCUSSION

Our results indicate that the majority *E. coli* strains enriched on the basis of their ability to proliferate in a seawater based medium, show a distinct growth response towards increased salinity. This observation is in agreement with results of Jones & Betaieb (1984) who showed that marine sediment impacted by sewage disposal contains up to 50% salinity-tolerant bacterial strains, mainly *Klebsiella*. Furthermore, the seawater *E. coli* isolates that grow at 7 °C are clearly distinct from other *E. coli* strains in this respect, since *E. coli* normally has a more restricted range of growth temperatures (Neidhardt *et al.* 1990).This agrees with data of Hänninen *et al.* (1995) who found lower maximum growth temperature ranges in *Aeromonas* from environmental sources. In consequence, our results suggest that the marine environment both in terms of salinity and low temperature, presents conditions that favour the selection of a particular group of *E. coli* strains.

On the other hand, about 6% of the *E. coli* strains we isolated from the marine environment contain transferable drug-resistance determinants, an observation consistent with earlier results of Smith (1971) who reported significant numbers of R-factor containing *E. coli* in coastal waters and of Shaw & Cabelli (1980) who found that about 5% of *E. coli* strains from an estuarine environment carry R-factors that are transmissible to and *E. coli* recipient.

More recently, transmission of plasmids from *E. coli* to natural marine bacteria has been reported (Robeson *et al.* 1990, Sorensen 1992). In addition, Goodman *et al.* (1993) have shown the feasibility of plasmid transmission, in crosses involving *E. coli*, under marine conditions. Therefore, the data regarding transmission of plasmid pUCV5, suggest that strains like *E. coli* 022 could probably donate R-plasmids to marine bacteria in the environment, particularly in sediments. In this respect, it would be advisable to rationalize the use of antibiotics for the control of pathogens in aquaculture, avoiding those which could represent a selective pressure for the emergence of resistant bacterial strains, a situation that has been described for fish pathogens (Aoki 1988).

With regard to the resistance determinants encountered, we noted that Hg-resistance is common among the resistant *E. coli* isolates we examined. Since this marker is common in *Pseudomonas* from the aquatic enviroment (Fry & Day 1990), it might be interesting to test the posibility that the Hg-resistance in *E. coli* isolates might represent the outcome of gene transfer from indigenous marine bacteria, since, in this regard, transfer of Hg-resistance from marine *Pseudomonas* to *E. coli* has been shown in laboratory experiments (Gauthier *et al.* 1985).

In summary, we have found that *E. coli* strains which survive in the marine environment and display the potential to proliferate therein also carry resistance determinants that represent a gene pool that might contribute to the genetic variability of autochthonous marine bacteria.

ACKNOWLEDGEMENTS

We thank FONDECYT (Grant nº 552-91) and the Dirección General de Investigación y Postgrado, Universidad Católica de Valparaíso for financial support.

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Recibido en julio de 1998 y aceptado en noviembre de 1998