Naturally-occurring, osmo-remedial variants of *Escherichia coli*

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Summary. Two clones of *Escherichia coli* O27:K1:H31 and O2:H7, isolated from patients with urinary tract infection or bacteraemia, failed to grow in a synthetic minimal medium (MM) of low osmolality. They were considered to be osmo-remedial because they grew well when sufficient amounts of NaCl, mannitol or sucrose were added to raise the osmolality of the medium to > 300 mOsm/kg. The defect could also be corrected by nicotinamide or its precursors quinolinic and aspartic acids. Each clone had a unique DNA restriction enzyme profile, fimbriae and antibiotic susceptibility patterns. The osmo-remedial variants were unstable and underwent phenotypic modulation to form mixtures with osmo-tolerant forms when grown in MM. They tended to form satellites of small colonies around large colonies of osmo-tolerant cells on MM agar plates. The penicillin method of Davis was used to separate the two forms. Nicotinamide induced the expression of *ompF* when the osmo-remedial strains were grown under conditions of low osmolality. It is possible that the variants are defective in the synthesis of membrane-derived oligosaccharides or outer-membrane proteins, but this has yet to be determined.

Introduction

Most strains of *Escherichia coli* can grow in synthetic minimal media containing salts, trace metals and a simple source of carbon and nitrogen such as glucose and ammonium. They can tolerate changes in osmolality of the medium in the range 150–1200 mOsm/kg.¹ Naturally-occurring auxotrophic variants have been isolated from the faeces of healthy individuals and from patients with urinary tract infections (UTI), bacteraemia and meningitis. The most common nutritional requirement is for nicotinamide^{2–4} but some strains require cysteine, thiamine, thymidine, glutamine or various amino acids.^{5,6}

During the course of a study of the salt tolerance of clinical isolates of *E. coli*, we encountered several strains of two serotypes, O27:K1:H31 and O2:H7, which failed to grow in minimal medium of low osmolality. They were regarded as osmo-remedial because they grew well when sufficient NaCl, mannitol, or sucrose was added to raise the osmolality of the medium to > 300 mOsm/kg. The defect could also be corrected by the addition of small amounts of nicotinamide. The strains were found to consist of mixtures of osmo-remedial and osmo-tolerant variants. This suggested that they might be undergoing phenotypic modulation.

We describe the isolation and characterisation of these strains. A variation of the penicillin method,

designed to isolate auxotrophic mutants of *E. coli*,⁷ enabled us to isolate the osmo-remedial variants, to study their osmotic and nutritional requirements and to characterise their outer-membrane proteins (OMPs).

Materials and methods

Bacterial strains

Clinical isolates of *E. coli* were obtained from 100 patients with bacteraemia, 101 young women with UTI, 100 rectal swabs from healthy individuals and 15 vaginal swabs from postmenopausal women. *E. coli* K10 was provided by Dr L. Tombras Smith of the University of California, Davis.⁸ *E. coli* ATCC 25922 was obtained from the American Type Culture Collection. Stock cultures, from a single colony of each isolate, were grown in Schaedler's Broth (Difco) containing glycerol 15% and stored at -40°C .

Media and chemicals

Minimal medium (MM)⁹ was composed of (/L) glucose 2 g, dipotassium phosphate 10.5 g, monopotassium phosphate 4.5 g, sodium citrate 0.5 g, magnesium sulphate 0.264 g and ammonium sulphate 1 g. The pH was adjusted to 7.2 before use. The osmolality was 231 mOsm/kg as determined by freezing point depression. Solid MM (SMM) was prepared by the

addition of Bacto-Agar (Difco) 2%. The batch of agar contained 0.34 mM NaCl/g, as determined at the Clinical Laboratories of the Ohio State University Hospital. In some experiments, the medium was supplemented with nicotinamide, nicotinic acid, quinolinic acid, aspartic acid, oxalacetic acid, glycine betaine, choline, thiamine, MEM non-essential and MEM essential amino acid mixtures, or ampicillin (Sigma). Other media or reagents included Trypticase Soy Broth (TSB, osmolality 301 mOsm/kg), Trypticase Soy Agar (TSA) (BBL Laboratories, Cockeysville, MD, USA), Mueller-Hinton Broth and penicillinase (β -lactamase) (Difco).

Growth conditions

A single colony from MacConkey agar was sub-cultured in MM and incubated overnight at 37°C. A 1 in 200 dilution of a 0.5 McFarland standard (final concentration $c. 5 \times 10^5$ cfu/ml) was added to tubes containing MM alone or with graded concentrations of NaCl (0.1–1 M in 0.1 M steps). Growth was assessed visually at 24 h and by optical density at 48 h with a Milton Roy spectrophotometer (Spectronic 601, Rochester, NY, USA) set at 600 nm. The end-point for salt tolerance was the maximum concentration of NaCl at which turbidity was $\geq 50\%$ (absorbance of $c. 0.5$) when compared with tubes in which there was full growth. In each experiment, *E. coli* strains K10 and ATCC 25922 were included as controls.

Serological typing and identification of P fimbriae

Strains were characterised by Dr C. Krishnan (Ontario, Canada Ministry of Health Laboratories) by O, K1 and H antigen and haemolysin production. P fimbriae were identified with latex particles to which α -D-Gal(-1-4)- β -D-Gal-disaccharide was covalently bonded (PF Test, Orion Diagnostica, Espoo, Finland).

Antimicrobial drug susceptibility

The tests were performed in standardised microwell dilution plates prepared by the Clinical Microbiology Laboratory of the Ohio State University.

Isolation of osmo-remedial E. coli with ampicillin and penicillinase

A modification of the penicillin method described by Davis⁷ was used to isolate the osmo-remedial strains. A 0.001-ml loopful of a single colony was picked from a TSA plate and incubated in TSB overnight at 37°C. The culture was centrifuged for 15 min at 5000 *g* and the pellet was washed three times with 10 ml of MM. The cells were adjusted to a 0.5 McFarland unit and then diluted 1×10^4 in MM containing ampicillin 100 mg/L. After incubation for 24 h at 37°C, 1000 units of penicillinase were added and the mixture was incubated for 4 h at 30°C. The

treated cells (0.1 ml) were added to tubes containing 0.9 ml of MM alone or MM with added NaCl, KCl, sucrose, mannitol, nicotinamide or other compounds. The tubes were incubated at 37°C and observed for turbidity daily for 5 days. Quantitative cultures were performed on TSA, just before addition of the ampicillin, 24 h later and 24 h after the penicillinase had been added.

Preparation of cell extracts for outer-membrane proteins (OMPs)

OMPs were prepared by differential centrifugation of crude sonicates obtained from overnight cultures grown in various media and purified by treatment with sodium-N-lauryl sarcosinate.¹⁰ They were resolved by vertical slab gel electrophoresis and stained with Coomassie Brilliant Blue.

DNA analysis

Bacterial chromosomal DNA was isolated according to the method of Van Ketel *et al.*¹¹ Briefly, bacteria were grown in TSB at 37°C in shaker flasks. Centrifuged pellets were washed in 10 mM Tris-HCl–10 mM disodium EDTA, pH 8.5, and treated with lysozyme 1 mg/ml and sodium dodecyl sulphate (Sigma) 1%. RNAase A 200 μ g/ml (Sigma) was added¹² and the solution was incubated overnight at 50°C with proteinase K (Sigma) 100 mg/L. After treatment with 1 M sodium perchlorate, the DNA was extracted with phenol–chloroform–isoamyl alcohol and precipitated with ethanol 95%. The DNA was digested with *EcoRI* (Gibco BRL, Gaithersburg, MD). Restriction enzyme digests were electrophoresed in agarose 0.3–0.5% in a Tris-acetate-EDTA buffer system¹³ with high-mol. wt DNA markers (Gibco BRL). Gels were stained in ethidium bromide 0.5 mg/L, destained in distilled H₂O and photographed under UV illumination.

Results

Isolation of osmo-remedial strains of E. coli

The clinical isolates of *E. coli* were screened for ability to grow in MM (231 mOsm/kg) alone, or in the presence of serial 0.1 M increments of NaCl. Five strains of *E. coli* O27:K1:H31 and one strain of O?:H31 failed to grow in MM by 48 h. Five of the six strains were isolated from patients with UTI and one was from a patient with bacteraemia. All the strains were relatively salt-tolerant (growing in 0.5–0.6 M NaCl), non-haemolytic and lacked P fimbriae. All but one strain were susceptible to representative β -lactam and aminoglycoside antibiotics and to co-trimoxazole. One strain was resistant to cephalothin. All of the strains were resistant to erythromycin > 4 mg/L and vancomycin > 16 mg/L.

Six strains of *E. coli* O2:H7 were osmo-remedial, failing to grow in MM without addition of NaCl. Two

Fig. 1. Restriction fragment analysis (*Eco*RI digest). Lane 1, M, markers; 2-7, different isolates of *E. coli* O27:K1:H31; 8, *E. coli* ATCC 25922; 9, *E. coli* K10; 10, *E. coli* O2:H7.

of these strains had been isolated from urine, two from blood cultures, one from stool and one from a vaginal swab. All the strains were relatively salt-tolerant (growing in 0.5–0.7 M NaCl), non-haemolytic and possessed P fimbriae. They varied in their susceptibility to antibiotics. Most were susceptible to all the antibiotics tested but two strains were resistant to ampicillin-sulbactam and amoxycillin-clavulanate. All were resistant to erythromycin > 4 mg/L and vancomycin > 16 mg/L.

DNA chromosomal analysis of the osmo-remedial strains

The restriction patterns obtained from *E. coli* O27:K1:H31 strains were similar to each other, but differed from those of *E. coli* ATCC 25922, K10 and O2:H7 (fig. 1). All strains of *E. coli* O2:H7 exhibited identical patterns.

Growth requirements of the osmo-remedial strains of *E. coli*

The strains were not killed when added to MM. They could be "rescued", and even after 3–5 days achieved full growth within 24 h after addition of any of several compounds, including various salts or sugars to an osmolality of 300 mOsm/kg and very low concentrations of nicotinamide, and some of its precursors (table I).

Initial attempts to isolate the osmo-remedial strains in pure culture

The osmo-remedial strains were, at times, found to grow in MM without added salt. This was noted even when single colonies were passed several times on SMM to avoid nutrient carry over. The effect appeared to be lost in some experiments or growth was delayed unless NaCl or nicotinamide was added. This was particularly common with several strains of *E. coli* O2:H7.

More consistent results were obtained when a low inoculum of $c. 1 \times 10^4$ cfu/ml was used. This led to the suspicion that each colony might be a mixture of osmo-remedial and osmo-tolerant cells. This notion was supported by experiments in which a single colony was picked from MacConkey agar, serially diluted and plated on SMM, or SMM with NaCl 0.2 M, or nicotinamide 0.1 mM. Uniform, large colonies were noted after incubation for 48 h. In a representative experiment there were 1.4×10^4 cfu/ml on SMM, 3.5×10^7 cfu on SMM + NaCl and 6.8×10^7 cfu on the SMM + nicotinamide. Thus, there were about 1000 times more osmo-remedial than osmo-tolerant colonies in the culture. Subcultures of colonies taken from SMM grew in MM and MM + 0.2 M NaCl, whereas those taken from SMM + NaCl grew only in MM + 0.2 M NaCl. It appeared as though osmo-remedial strains had been isolated in MM + NaCl and osmo-tolerant strains in plain MM. However, subcultures of the putative osmo-remedial isolates grew well when streaked on SMM and appeared to have reverted to osmotic tolerance.

Tiny colonies were observed to grow on SMM after about 72 h. They were not found on SMM containing NaCl or nicotinamide. Six sets of the tiny colonies were cut from the agar and subcultured in MM or MM + NaCl. Three of the sets grew in MM + NaCl only, whereas the other sets grew in MM or MM + NaCl. This experiment was repeated several times with similar results. Thus, it was not possible consistently to separate the osmo-remedial from the osmo-tolerant forms by sequential subculture in MM + NaCl and SMM + NaCl, by terminal dilution or by isolation of individual tiny colonies.

Use of ampicillin with penicillinase rescue to isolate osmo-remedial *E. coli*

This approach was based on the concept that the osmo-tolerant variants would grow in MM and be killed by ampicillin, whereas the osmo-remedial variants would not grow in MM and therefore not be killed. The osmo-remedial variants would then be rescued by addition of osmolytes or nicotinamide, once the ampicillin had been inactivated by penicillinase.

This method proved to be effective for the isolation of the osmo-remedial strains of *E. coli*. Two strains of *E. coli* O27:K1:H31, six strains of O2:H7 and the control strains of *E. coli* ATCC 25922 and K10 were

Fig. 2. Satellite phenomenon demonstrated on MMA. The surface of the plate was inoculated uniformly with an osmo-sensitive strain of *E. coli* O27:K1:H31 and then *E. coli* K10 was streaked across its diameter.

studied. Ampicillin (100 mg/L) reduced the inoculum of all strains in MM from $c. 1 \times 10^3$ cfu to $< 1 \times 10^1$ cfu within 24 h. After addition of penicillinase, the control strains of *E. coli* ATCC 25922 and K10 became turbid

Table I. Compounds that supported growth in MM of osmo-remedial strains of *E. coli* O27:K1:31 and O2:H7

Compound*	Minimal concentration that supported growth
NaCl	0.1 M
KCl	0.1 M
Mannitol	0.2 M
Sucrose	0.2 M
Nicotinamide	< 0.1 mM
Quinolinic acid	1.0 mM
Aspartic acid	0.1 mM†

* Glutamic acid, oxalacetic acid, glycine betaine, choline and thiamine were not active at 1 mM. A mixture of essential amino acids did not restore growth.

† Supported growth of *E. coli* O2:H7 only.

within 24–48 h. Tubes containing the osmo-remedial strains in MM alone remained clear for ≥ 5 days. Identical tubes which contained osmolytes, nicotinamide or its precursors became turbid within 1–2 days. The ampicillin–penicillinase method was less effective with larger inocula, smaller amounts of ampicillin (20 mg/L), or when the strains were grown in MM rather than TSB before addition of the ampicillin.

Satellite phenomenon with osmo-remedial strains

Despite the apparent success of the ampicillin–penicillinase method for the isolation of osmo-remedial variants, reversion to osmo-tolerance still occurred. This was observed when subcultures from MM containing NaCl, mannitol or nicotinamide were plated on SMM. A mixture of large and tiny colonies was observed on SMM even at a dilution of 1×10^{-7} . The tiny colonies tended to grow as satellites around large colonies.

To examine this phenomenon further, the surface of SMM was uniformly inoculated with *E. coli* O27:K1:H31 that had been exposed to ampicillin and

Fig. 3. SDS-PAGE of OMPs of *E. coli* O27:K1:H31 (a) and *E. coli* O2:H7 (b) grown in MM or TSB. Lanes 1 and 9, M_r markers; 2, MM with 0.2 M NaCl; 3, MM with 0.2 M NaCl and 1 mM nicotinamide; 4, MM with 0.4 M mannitol; 5, MM with 0.4 M mannitol and 1 mM nicotinamide; 6, TSB; 7, TSB with 1 mM nicotinamide; 8, MM with 1 mM nicotinamide. The arrows point to the increased amount of *ompF* noted in lane 8 for each strain.

Fig. 4. SDS-PAGE of OMPs of *E. coli* K10 grown in MM or TSB. Lanes 1 and 9, M_r markers; 2, MM with 0.2 M NaCl; 3, MM with 0.2 M NaCl and 1 mM nicotinamide; 4, MM with 0.4 M mannitol; 5, MM with 0.4 M mannitol and 1 mM nicotinamide; 6, TSB; 7, TSB with 1 mM nicotinamide; 8, MM with 1 mM nicotinamide; 8A, MM alone.

penicillinase. The plate was then streaked across its diameter with 0.001 ml of an osmo-tolerant strain of *E. coli* K10 grown in MM (fig. 2). The satellites seen in the region of the streak indicate that nutrients had diffused from the *E. coli* K10 to support growth of the osmo-remedial strain.

Characterisation of OMP proteins of the osmo-remedial strains

Representative strains of *E. coli* O27:H7 and K10, which had been treated with ampicillin and penicillinase, were incubated for 24 h at 37°C in MM containing 0.2 M NaCl, 0.4 M mannitol or 1 mM nicotinamide or in TSB with and without nicotinamide. The OMPs were harvested and identified by gel electrophoresis (fig. 3). The most striking finding was the consistent presence of a dense band in the region of *ompF* when the osmo-remedial strains were grown in MM containing 1 mM nicotinamide. This effect was not observed with cells grown in MM + NaCl, mannitol or TSB with or without added nicotinamide. Nicotinamide had no discernible effect on the OMP patterns of *E. coli* K10 (fig. 4).

Discussion

Naturally-occurring, osmo-remedial *E. coli* have not, to our knowledge, been reported previously. The two osmo-remedial clones of *E. coli* described here differed from each other in serotype, fimbrial characteristics, antibiotic susceptibility and chromosomal DNA patterns. The mechanisms that account for the inability of the osmo-remedial variants to grow at low

osmolality and their reversion to osmo-tolerant forms are not entirely clear. Several possibilities may be considered.

Firstly, *E. coli* responds to low osmolality by increasing the synthesis of membrane-derived oligosaccharides (MDO)¹⁴ and the *ompF* porin.¹⁵⁻¹⁷ MDO is localised in the periplasm and thought to be one of several mechanisms by which gram-negative bacteria cope with low osmolality; *ompF* permits the uptake of relatively large hydrophobic or negatively-charged compounds.^{18,19} Mutants of *E. coli* unable to synthesise MDO exhibit decreased expression of *ompF* when grown in medium of low osmolality and ionic strength.²⁰ It has been proposed that a minimal ionic strength in the periplasm is necessary for normal porin regulation.²¹

It is possible that the osmo-remedial strains are unable to respond to low osmolality by synthesis of MDO and *ompF*. This is supported, in part, by the finding that *ompF* was increased by nicotinamide under hypotonic conditions. The effect was abolished when the osmolality of the medium was increased by adding NaCl or mannitol.

Secondly, Env A permeability mutants of *E. coli* which allow periplasmic β -lactamase and RNAase I to leak into the medium have been described.²² These mutants are highly susceptible to a wide variety of antibiotics including erythromycin and vancomycin. It is doubtful whether the osmo-remedial strains have a defect since they were resistant to these drugs.

Finally, a critical osmolality, ionic strength or temperature is often necessary for the phenotypic expression of enzyme activity in mutant bacteria.²³ Temperature-sensitive mutants of *E. coli*²⁴ and salt-correctable, temperature-sensitive, histidine auxotrophs of *Salmonella typhimurium*²⁵ have been described. There are also reports of osmo-remedial adenine- and glutamate-requiring, and ionic strength-remedial pantothenate mutants of *Neurospora crassa*.²⁵⁻²⁷ Similar effects have been described in auxotrophic mutants of yeasts.²⁸

Phase variation and phenotypic modulation is fairly common among micro-organisms. For example, expression of type I fimbriae in *E. coli* depends on the liquid or solid nature of the medium.²⁹ In *Bordetella pertussis*, the virulent-phase genes are reversibly regulated by temperature, high concentrations of $MgSO_4$ and nicotinic acid.³⁰ NaCl also effects the expression of heterogeneous methicillin-resistant *Staphylococcus aureus*.³¹ In view of these observations, it is reasonable to conclude that the osmo-remedial variants exhibit a form of phenotypic modulation, induced by an increase in the osmolality of the medium, which permits them to synthesise nicotinamide from precursors of aspartic or quinolinic acids.

The osmo-remedial, nicotinamide-requiring strains do not appear to have a disadvantage for survival in nature, since they were readily isolated from clinical specimens. This may be due to the relative abundance of nicotinamide in body fluids³² or because protective

compounds may be derived from other bacteria growing in the same environment.

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