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M.W. RHODES AND H. KATOR. 1997. A modified mE medium (mEI) containing the chromogenic substrate indoxyl-β-D-glucoside to detect β-D-glucosidase activity was evaluated with respect to specificity and recovery of enterococci from environmental waters. Extending incubation from 24 to 48 h improved enterococci recovery but 77% of the colonies classified as non-target were confirmed as enterococci. Randomly chosen enterococcal isolates from sewage, exposed in microcosms containing 0·22 μm membrane filtered fresh or estuarine water, exhibited differences in persistence as a function of exposure treatment. Decreasing the concentration of or eliminating indoxyl-β-D-glucoside from mE did not significantly affect recovery of purified isolates.

INTRODUCTION

Concerns related to the validity of faecal coliforms as indicators have prompted interest in the use of enterococci as predictors of enteric disease risk associated with environmental waters. Retrospective epidemiological studies have documented relationships between enterococcal densities and swimming-associated gastrointestinal disease in both fresh and marine waters (Cabelli *et al.* 1983). Consequently, the US Environmental Protection Agency (Anon. 1986) established recreational freshwater quality criteria that specify geometric mean densities not to exceed 33 enterococci or 126 *Escherichia coli* per 100 ml. Enterococci are also clinically significant opportunistic pathogens causing bacteriuria, bacteraemia, wound sepsis and subacute bacterial endocarditis, infections now complicated by the increased resistance of enterococci to antimicrobial agents (Murray 1990; Facklam and Washington 1991).

The approved method (Anon. 1985, 1993, 1995) for enterococci enumeration is a two-step procedure: growth on a selective and differential medium (mE) for 48 h followed by an *in situ* test for aesculin hydrolysis (Levin *et al.* 1975). Aesculin (6,7-dihydroxycoumarin 6-glucoside) is hydrolysed by a β-glucosidase to glucose and esculetin which in the presence of ferric ions produces a brownish black precipitate (Anon. 1994). The use of chromogenic and fluorogenic substrates for detection of glycosidic activity to differentiate enterococci has received considerable attention (Dufour 1980; Littel and Hartman 1983; Pourcher *et al.* 1991; Hernandez *et al.* 1991, 1993; Manafi and Sommer 1993). Dufour (1980) proposed a modification of the mE method to a 24 h one-step procedure by eliminating the *in situ* aesculin test through substitution of indoxyl-β-D-glucoside for aesculin in the primary medium. Bacteria growing on media containing an indoxyl-β-D-glycoside and producing the appropriate glycosidase will form blue colonies through release of an aglycone that is converted to indigo (Ley *et al.* 1988).

Typically, fluorogenic or chromogenic glycosides are added to media that already contain other carbohydrates (Feng and Hartman 1982; Littel and Hartman 1983; Watkins *et al.* 1988; Pourcher *et al.* 1991; Hernandez *et al.* 1991, 1993; Manafi and Sommer 1993). Adding a test compound to a commercially produced medium is expedient owing to the ease and cost of preparation and facilitates quality control. Consequently, the present study was undertaken to evaluate enumeration of enterococci using a commercial mE medium amended with indoxyl-β-D-glucoside. Specifically, a modified mE medium (mEI) was examined with regard to (1) the effect of incubation time on detection of target colonies, (2) specificity, and (3) influence of sublethal stress on enterococcal recovery.

Taxonomy of the genus *Enterococcus* (Schleifer and Kilpper-Balz 1984) has been revised and new species recognized (Devriese *et al.* 1983; Farrow *et al.* 1984; Williams *et al.* 1989; Rodrigues and Collins 1990; Devriese *et al.* 1990; Collins *et al.* 1991; Kusuda *et al.* 1991; Martinez and Collins 1991). Speciation of enterococcal isolates can be significant in assessing their faecal or non-faecal origin (Colman *et al.* 1992).

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Accordingly, we examined the specificity of the modified mE method applied to environmental samples using differential criteria (Facklam and Collins 1989; Collins *et al.* 1991; Martinez-Murcia and Collins 1991) that provide for identification of 15 *Enterococcus* sp. which hydrolyse aesculin aerobically and react with group D antisera.

Finally, in view of the recommended use of enterococci as the basis for health risk criteria applicable to both fresh and marine waters (Anon. 1986), recovery and extent of sublethal stress were measured for environmental enterococcal isolates following exposure in microcosms to filtered fresh or estuarine water.

MATERIALS AND METHODS

Sample collection

Sixty-two water samples were collected over a period of 7 months from two eutrophic coastal plain freshwater lakes in Virginia (Lake Powell and Lake Matoaka), previously characterized with respect to a variety of bacteriological and chemical parameters (Rhodes and Kator 1994), and from Lake Matoaka feeder streams. Enterococci isolated from these sources were approximately equally represented by lake (47%) and stream (51%) origins. Additionally, 23 feeder stream samples were collected to examine the effect of extended incubation time on counts of enterococci. Samples were collected in sterile containers using an aseptic technique, held in an insulated container during transport, and processed within 4–6 h after collection.

Escherichia coli enumeration

Samples were inoculated into lactose broth (Difco Laboratories, Detroit, MI) as the presumptive medium and EC broth (Difco) as the confirmatory medium using a five-tube MPN procedure (Anon. 1995). The fluorogenic substrate, 4-methylumbelliferyl-β-D-glucuronide (MUG; Sigma, St Louis, MO), was incorporated into EC broth to estimate *E. coli* densities (Feng and Hartman 1982).

Enterococci enumeration

Either quadruplicate 25 ml or triplicate 10 ml and 1 ml aliquots of water samples were processed by membrane filtration (Anon. 1995). Membranes were transferred to commercial mE medium (Difco) amended with 75 mg of indoxyl-β-D-glucoside 100 ml^{-1} (Dufour 1980), hereafter called mEI medium. Filtered samples collected at water temperatures below 20°C were resuscitated for 2–3 h at room temperature (*ca* 24°C) before incubation at 41°C. Preliminary results showed that extending incubation from 24 to 48 h increased the number of presumptive target colonies and improved colour development. Thereafter, target colonies counted were those that produced indigo blue by hydrolysis of indoxyl-β-D-glucoside (Bisson and Cabelli 1979) after 48 h incubation. Colonies were counted using a stereoscopic microscope, replicate counts averaged, 95% confidence intervals calculated, the means log transformed and experimental treatments compared using Student's paired sample *t*-test.

Isolate identification

Target blue and non-target colonies were randomly selected from environmental water samples processed on mEI medium after 48 h incubation. Isolates were purified on brain heart infusion (BHI) agar (Difco) and identified using published criteria and methods (Facklam and Collins 1989; Collins *et al.* 1991; Martinez-Murcia and Collins 1991). Isolates were also examined for starch hydrolysis, liquefaction of 12% gelatin, and reaction in litmus milk (Difco) (Facklam and Washington 1991; Anon. 1994).

Enumeration of enterococci exposed to filtered fresh and estuarine water in microcosms

A sample of raw sewage was processed using the approved mE method (Levin and Cabelli 1975) and five randomly selected presumptive enterococci isolates were selected for purification and confirmation as enterococci (Anon. 1993). Confirmed enterococci isolates were identified to species and their culturability determined after exposure to membrane-filtered fresh and estuarine water. Each isolate was grown overnight in BHI broth and inoculated into filtered (0·22 μm) Lake Matoaka or York River (20 psu) water to yield *ca* 10^{3-4} cells ml^{-1}. Cell densities were determined immediately after inoculation and after 3 weeks static incubation in the dark (13°C). Enterococci were enumerated by membrane filtration on mE medium containing either 0, 7·5 or 75 mg of indoxyl-β-D-glucoside 100 ml^{-1} and BHI agar as a non-selective control medium. Colony counts were determined at 24 and 48 h incubation as described above using triplicate plates for each dilution required and the 95% confidence limits of these means calculated. The Student's *t*-test was used to compare log transformed colony counts for the two incubation periods. Differences between recovery on BHI and mE-based media were determined by the Friedman test for analysis of variance by ranks (Zar 1984). Differences in persistence as a function of exposure treatment, i.e. estuarine *vs* freshwater, and between enumeration media were tested using a non-parametric two-way ANOVA based on the Kruskal–Wallace test (Zar 1984).

RESULTS

Effect of incubation period

Enterococci counts on mEI from seasonal stream water samples were compared after 24 and 48 h incubation with respect

Table 1 Effect of incubation time on recovery of enterococci from freshwater stream samples using mEI medium

Date	Sample site	*E. coli* 100 ml^{-1}	Number of colonies 100 ml^{-1} after incubation for: 24 h Target	24 h Non-target	48 h Target	48 h Non-target
March	B	1100	<1	ND	41	19
	C	170	<1	ND	14	2
	D	130	<1	ND	7	2
	E	790	<1	ND	17	1
	F	49	<1	ND	5	<1
	Mean*	250	<1	ND	13	2
June	A	49	<1	44	92	84
	B	490	<1	60	112	83
	C	220	<1	180	260	87
	D	330	<1	4	16	3
	E	9200	<1	282	644	61
	F	47	<1	9	24	<1
	Mean	300	<1	41	93	22
August	A	280	<1	<1	1	<1
	B	350	<1	2	10	1
	C	79	<1	3	6	<1
	D	330	1	<1	2	<1
	E	790	<1	<1	3	37
	F	490	<1	<1	1	1
	Mean	320	<1	1	3	2
February	A	310	<1	44	397	114
	B	68	<1	118	206	40
	C	330	<1	18	68	400
	D	130	<1	15	76	6
	E	110	<1	24	87	17
	F	460	<1	30	245	39
	Mean	190	<1	32	144	44

ND, Not determined.
* Mean of log transformed data.

to colour development (Table 1). Blue target colonies were frequently surrounded by a blue halo. Non-target colonies ranged in colour and intensity from pink to maroon. Extended incubation (48 h) yielded significant increases (Student's t, $P < 0{\cdot}001$) in target colony counts but did not affect counts of non-target colonies (Student's t, $P > 0{\cdot}05$). Increases in enterococci densities during prolonged incubation occurred with samples representing contrasting seasonal conditions (June and February).

Specificity of mEI medium

Target blue colonies were identified primarily as *Ent. casseliflavus* or *Ent. faecium* and less frequently as *Ent. faecalis* and *Ent. hirae* (Table 2). Negative pyrrolidonylarylamidase (PYR) reactions were observed in 6% of the target blue colonies. Approximately 77% of the non-target colonies isolates were identified as enterococci. The relative dominance of enterococcal species comprising non-target colonies was similar to target colonies. More than 50% of the isolates yielding a negative bile aesculin reaction formed small (<0·5 mm diam.) red colonies.

Persistence and recovery of culturable *Enterococcus* sp. in filtered lake and estuarine water

The persistence of five enterococcal sewage isolates was superior in estuarine water compared with freshwater (Table 3). Differences in persistence of culturable cells between

Table 2 Identification of isolates recovered from freshwater samples enumerated on mEI medium

Characterized as :	Number of isolates*	
	Target	Non-target
Enterococcus casseliflavus	49 (28)	23 (20)
Ent. durans	5 (3)	2 (2)
Ent. faecalis	25 (14)	8 (7)
Ent. faecium	49 (28)	34 (30)
Ent. gallinarum	5 (3)	0 (0)
Ent. hirae	22 (13)	8 (7)
Ent. mundtii	8 (5)	13 (11)
BE-negative†	0 (0)	16 (14)
PYR-negative‡	11 (6)	10 (9)
BE- and PYR-negative	0 (0)	1 (<1)
Total isolates tested	174 (100)	115 (100)

* Value in parentheses indicates per cent.
† Negative bile aesculin reaction.
‡ Negative pyrrolidonylarylamidase reaction.

isolates occurred with both maximum and minimum recoveries shown by strains of *Ent. faecalis*. After 3 weeks exposure, log reductions in numbers of cells ranged from 0·6 (*Ent. faecalis* isolate 1) to 3·5 (*Ent. faecalis* isolate 3) in lake water and from 0·3–0·4 (*Ent. faecalis* isolate 1, *Ent. faecium*, *Ent. hirae*) to 1·1 (*Ent. faecalis* isolate 3) in estuarine water. Differences in persistence as a function of exposure treatment and enumeration media were tested using a non-parametric two-way ANOVA test. Results were significant for exposure conditions, i.e. estuarine *vs* freshwater ($P < 0{\cdot}001$), and not significant for media type ($P > 0{\cdot}05$).

There was variation among isolates when recovery on BHI was compared to selective mE-based media. Following exposure to estuarine water, *Ent. faecalis* isolates 2 and 3 showed decreased recovery on all of the mE-based media compared to BHI. However, considering the entire dataset, there was no significant difference between count data on BHI and mE media containing 0, 7·5 or 75 mg 100 ml^{-1} (Friedman test, $P > 0{\cdot}05$).

Colony counts on mEI medium containing 75 mg 100 ml^{-1} indoxyl-β-D glucoside, after 24 and 48 h incubation were not

Table 3 Persistence and recovery of *Enterococcus* sp. on selective and non-selective media after *in vitro* exposure to fresh (F) and estuarine (E) water at 13°C

Isolate	Exposure	Persistence* (%)	Recovery as culturable cells ml^{-1}†			
			BHI	Indoxyl-β-D-glucoside (mg 100 ml^{-1}) in mE medium		
				0	7·5	75
Ent. faecalis						
1	F	23·8	4960 ± 15	5320 ± 14	5110 ± 97	5320 ± 61
	E	52·2	11 840 ± 140	10 200 ± 190	10 300 ± 160	10 720 ± 140
2	F	1·7	550 ± 14	610 ± 11	530 ± 9	600 ± 27
	E	15·2	4670 ± 11	1870 ± 57	1790 ± 24	1760 ± 43
3	F	0·0	4 ± 0·8	2 ± 0·2	2 ± 0·8	2 ± 0·7
	E	8·2	1150 ± 24	560 ± 12	410 ± 8	450 ± 26
Ent. faecium						
	F	23·3	1380 ± 17	1380 ± 33	1240 ± 31	1300 ± 42
	E	40·4	1820 ± 28	1360 ± 17	1530 ± 13	1580 ± 28
Ent. hirae						
	F	11·2	1860 ± 39	1630 ± 27	1620 ± 9	1690 ± 16
	E	48·9	8780 ± 40	8820 ± 26	9370 ± 78	8970 ± 94

* Persistence following 3 weeks exposure expressed as the per cent ratio of culturable cell counts enumerated on brain heart infusion agar (BHI) after 48 h incubation to those at 0 time.
† Recovery of exposed cells based on mean of culturable cells enumerated by membrane filtration on BHI and mE media after 48 h incubation ± 95% confidence limits.

distinguishable for isolates exposed to estuarine or freshwater (Student's t, $P > 0{\cdot}05$). Qualitatively, however, *Ent. faecalis* isolates 2 and 3 and *Ent. hirae* showed marked variation in size, colour and halo development on mEI.

DISCUSSION

Two performance characteristics critical to the evaluation of candidate indicator methods, especially when applied to environmental samples, are efficiency and specificity, i.e. occurrence of false positives and false negatives (Kator and Rhodes 1994). Indicator densities can be underestimated because environmentally stressed cells fail to grow under selective cultural conditions or to express a critical phenotypic characteristic. Moreover, the specificity of a method can be 'affected' by revisions in bacterial taxonomy based on 16S rRNA and DNA–DNA hybridization studies. Because of taxonomic revision, historical reliance on a limited number of biochemical tests may no longer lead to correct identification of target and non-target colonies.

The occurrence of sublethal injury in indicator bacteria including the enterococci has been extensively reviewed (e.g. McFeters 1989; Ray 1989). In the present study improved recovery of verifiable enterococci from water samples with extended incubation and the significant proportion of enterococcal colonies which either did not hydrolyse or weakly hydrolysed indoxyl-β-D-glucoside indicated sublethal stress. Failure to detect stressed enterococci would underestimate their densities. All environmental water samples examined (Table 1) were acceptable based on the US EPA enterococci water quality criterion of < 33 enterococci 100 ml^{-1} (Anon. 1986) determined after 24 h incubation on mEI medium. Extending the incubation period to 48 h increased enterococci counts in nearly every sample and in 11 cases the EPA criterion was exceeded. In comparison the recreational water criterion of 126 *E. coli* 100 ml^{-1} was exceeded in 18 of the 23 same water samples (Table 1).

In contrast to environmental samples, recovery of laboratory isolates exposed to filtered natural waters was not improved by an extended incubation period. Niemi and Ahtiainen (1995) reiterated that laboratory-adapted isolates can be more refractive to harsh cultivation conditions than target bacteria in natural samples and that an evaluation of enumeration methods should use recovery data from both pure cultures and natural samples. Niemi and Ahtiainen (1995) also observed that recovery of laboratory strains of *Enterococcus* sp. varied as a function of enumeration media. Our observations that isolates of the same species exhibited different persistence and recovery characteristics on selective media following exposure to fresh and estuarine water support Niemi and Ahtiainen (1995). Variations in colony morphology (e.g. size, colour, presence of blue halo) also occurred when a microcosm-exposed isolate was plated on mEI.

A variety of chemical agents present in mEI medium could singly or in concert affect recovery of environmentally stressed cells. Although sodium azide is frequently used in enterococcal media to inhibit Gram-negative organisms, enterococci are sensitive to sodium azide as a function of concentration (Ramadan 1968; Barnes 1976) and extent of stress (Ray 1989). Other selective components in mE medium include nalidixic acid for inhibition of Gram-negative organisms and actidione to retard fungal growth. Triphenyltetrazolium chloride (TTC) in culture media is reduced by enterococci at pH 7·0 to an insoluble red formazan facilitating detection of target colonies (Barnes 1976). Toxicity of TTC to Gram-positive bacteria (Weinberg 1953) and to some faecal streptococci (Donnelly and Hartman 1978) at concentrations exceeding 0·001% has been reported. Variation in resistance to higher concentrations of TTC, i.e. 0·01 and 0·25%, is also a function of enterococci species examined (Facklam and Collins 1989; Kusuda *et al.* 1991). A recent study suggests that reducing the TTC concentration from 0·015% to 0·002% in another modification of mE medium containing the indoxyl-β-D-glucoside yields an effective 24 h enumeration procedure for enterococci (A. Dufour, US Environmental Protection Agency, Cincinnati, OH, USA; personal communication).

Indoxyl-β-D-glucoside has been reported inhibitory to another Gram-positive species. Armon and Payment (1988) observed improved recovery of *Clostridium perfringens* from sewage when the glucoside concentration in mCP medium was reduced from 0·06% to 0·006%. Similarly, we observed reduced recovery of *Cl. perfringens* from sewage-contaminated shellfish as a function of glucoside concentration in mCP in our laboratory (unpublished results). However, observations reported in the present study indicated recovery of purified enterococci was not improved by eliminating or reducing the concentration of the chromogenic substrate. Additional experiments are necessary to evaluate the effect of indoxyl-β-D-glucoside on enumeration of enterococci in natural samples.

Another factor possibly affecting enterococcal detection is addition of the indoxyl-β-D-glucoside to mE which as formulated contains the substrate aesculin, a glucoside with a different aglycon moiety. Although preferred hydrolysis of one of the glucosides could occur, generally differences in aglycon specificity result in reduced rates of hydrolysis; rarely is hydrolysis completely inhibited (Baumann and Pigman 1957). End product inhibition of glucosidase by glucose is also possible (Baumann and Pigman 1957; Larner 1960). However, Littel and Hartman (1983) noted that the presence of a glycoside with an aglycon group and its end product derivative, i.e. methylumbelliferone arabinose and arabinose, did not inhibit hydrolysis of the fluorogenic compound and that incorporation of galactose and methylumbelliferone galactoside into a solid medium actually enhanced colony size, counts and fluorescent intensity. In general, addition of fluo-

rogenic or chromogenic labelled substrates to media containing alternate monosaccharides or glycosides (Feng and Hartman 1982; Littel and Hartman 1983; Watkins *et al.* 1988; Pourcher *et al.* 1991; Hernandez *et al.* 1991, 1993; Manafi and Sommer 1993) has been effective. These observations suggest that presence of an alternative carbohydrate does not adversely affect visualization of the chromophore or fluorophore.

Various workers have noted that although the original two-step mE method is very specific, e.g. low occurrence of false positives, enterococcal recoveries are lower compared with other procedures (Dutka and Kwan 1978; Pagel and Hardy 1980). Large false-negative rates have also been observed for the original mE procedure for samples of sewage and sewage effluent (73%, Pagel and Hardy 1980), and in our laboratory for sewage-contaminated shellfish (80%, unpublished data). Our finding that 77% of non-target isolates were enterococci, indicated the modified mE procedure also underestimated enterococcal densities even when the incubation period was extended to 48 h. Speciation of presumptive target colonies revealed that non-faecal enterococci, *Ent. casseliflavus* and *Ent. mundtii*, occurred at a combined frequency of 33% in environmental water samples.

An evaluation of medium specificity can be affected by taxonomic revisions. Standard criteria to verify an isolate as belonging to the 'enterococcal' group include a positive Gram stain reaction, growth at 45°C and in 6·5% NaCl, hydrolysis of aesculin in the presence of bile, and supplemental observations of reactions with Lancefield Group D antisera (Anon. 1993, 1995). However, based on recent taxonomic revisions at least six *Enterococcus* sp. would not be validated: *Ent. cercorum*, isolated from the caeca of chickens (Williams *et al.* 1989); *Ent. columbae*, derived from the intestines of pigeons (Devriese *et al.* 1990); *Ent dispar*, isolated from humans (Collins *et al.* 1991); *Ent. saccharolyticus*, obtained from cows and straw bedding (Rodrigues and Collins 1990); *Ent. seriolicida*, a marine and freshwater fish pathogen (Kusuda *et al.* 1991); and *Ent. sulfureus*, a yellow-pigmented species associated with plants (Martinez-Murcia and Collins 1991). Hydrolysis of pyrrolidonyl-β-naphthylamide (Schleifer and Klipper-Balz 1984) is now the basis for rapid identification of enterococci (Bosley *et al.* 1983; Manafi and Sommer 1993) although this reaction is not observed for some *Enterococcus* sp.: *Ent. cecorum* (Williams *et al.* 1989), *Ent. columbae* (Devriese 1990) and *Ent. saccharolyticus* (Rodrigues and Collins 1990). Therefore, in the present study it cannot be assumed that PYR-negative isolates were not enterococci as phenotypic features for differentiating PYR-negative enterococci from *Streptococcus bovis* (Devriese *et al.* 1990) were not included. However, all PYR-negative isolates recovered on modified mE were also starch positive, a characteristic that is consistent with an identification of *Strep. bovis*.

Our results demonstrated the importance of sublethal stress studies in evaluating methods for recovery of target bacteria. Based on recovery from environmental samples, mE medium augmented with indoxyl-β-D-glucoside was not an effective 24 h enumeration method. Although recovery was improved by extending incubation to 48 h, manipulation of other medium components, perhaps TTC, could produce an improved 24 h enumeration method. Importantly, future studies should include both pure cultures of target and non-target bacteria and environmental samples.

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