FACTORS AFFECTING THE GROWTH OF ENTEROCOCCI IN HIGHLY ALKALINE MEDIA[1]

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The ability to grow in a medium at pH 9.6 was one of the tolerance tests suggested by Sherman and Stark (1934) for the identification of *Streptococcus faecalis*. Subsequent investigations have indicated that the ability to grow at this high pH is a general characteristic of the enterococcus group with the exception of some strains of *Streptococcus durans* (Smith and Sherman, 1938; Shattock and Hirsch, 1947; Shattock, 1955). Shattock and Hirsch (1947) pointed out some of the experimental difficulties in determining this characteristic and devised a liquid medium buffered with glycine that standardized the conditions of the test and gave more reproducible results. However, this laboratory has experienced some difficulty in obtaining consistent results with the method of Shattock and Hirsch. Skadhauge (1950) used a broth medium adjusted to pH 9.6 with sodium carbonate, apparently with good results. The present study has clarified some of the factors that may lead to erratic results with these tests.

MATERIALS AND METHODS

Much of the work has been conducted with 5 cultures representing one strain each of *S. faecalis*, *S. faecalis* var. *liquefaciens*, *S. faecalis* var. *zymogenes*, *Streptococcus faecium* (Lake *et al.*, 1957), and *S. durans*. A number of tests also utilized a collection of 50 enterococci representing a variety of natural habitats and geographical sources.

Cultures were generally carried in tryptone-yeast-glucose broth of the following composition: 1.0 per cent tryptone, 0.5 per cent yeast extract, 1.0 per cent glucose, 0.25 per cent K_2HPO_4, and 0.25 per cent NaCl. This medium also served as the basal medium for most of the tests.

The basal medium, less the K_2HPO_4, was autoclaved for 10 min at 4× concentration. Test substances and the K_2HPO_4 were autoclaved separately at 4× concentration and were added aseptically to the basal medium. The pH was adjusted with sterile 2 N NaOH or 2 N H_3PO_4 and checked with the glass electrode, after which the medium was diluted to single strength with sterile distilled water and dispensed into screw capped test tubes. Inocula were always brought to 30 C at least 1 hr before use, and the inoculated medium was incubated at 30 C. Growth was measured turbidimetrically at 640 mμ with a Bausch and Lomb Spectronic-20. Uninoculated tubes of the medium were used as reference blanks throughout the experiment and their pH was checked at the termination of the experiment.

The pH was determined on a Beckman Zeromatic pH meter, using a shielded glass electrode. The instrument was standardized at pH 9.18 against 0.01 M $Na_2B_4O_7 \cdot 10H_2O$ and at pH 10.02 against a mixture of 0.025 M $NaHCO_3$ and 0.025 M Na_2CO_3. Cross reference between the standard buffers indicated a deviation of ±0.02 units in this range.

RESULTS AND DISCUSSION

Carbonate stimulation. The enterococci were found to grow poorly or not at all after short incubation periods in the tryptone-yeast-glucose broth at a pH above 9.0 unless sodium carbonate was used as the buffer. Between pH 8.0 and 9.0 added carbonate was strongly stimulatory. Table 1 illustrates these effects. Consequently, in all subsequent experiments the media at pH values above 8.0 contained 0.05 M Na_2CO_3 that had been sterilized by autoclaving a 0.20 M solution in a tightly capped bottle and aseptically added to the basal medium. The finding of this carbonate requirement may explain some of the difficulties encountered with the medium of Shattock and Hirsch (1947). When prepared exactly according to the originally described procedure,

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TABLE 1

Effect of sodium carbonate on growth of enterococci at alkaline pH levels

Organism	Na_2CO_3 Conc	pH 8.0	pH 8.5	pH 9.0	pH 9.6
	M				
Streptococcus durans	0	79*	59*	20*	0*
	0.005	88	79	60	60
	0.01	94	85	71	100
	0.05	80	64	66	100
Streptococcus faecalis var. *liquefaciens*	0	76	32	11	0
	0.005	122	119	75	22
	0.01	130	130	98	53
	0.05	126	122	116	135
S. faecalis	0	85	60	0	0
	0.005	118	122	92	60
	0.01	119	126	113	89
	0.05	118	119	116	140

* Growth was measured as optical density × 100 at 640 mμ after incubation at 30 C for 11 hr (pH 8.0, 8.5, and 9.0) or 14 hr (pH 9.6).

TABLE 2

*Toxicity of glycine for a typical enterococcus in an alkaline growth medium**

Time of Incubation at 30 C	pH 7.0		pH 8.3		pH 9.6	
	Tryptone-yeast-glucose		Tryptone-yeast-glucose		Tryptone-yeast-glucose	
	No addition	+ 0.05 M Glycine	No addition	+ 0.05 M Glycine	No addition	+ 0.05 M Glycine
hr						
6	0	0	0	0	0	0
8	8	8	8	3	0	0
10	47	64	59	37	0	0
12	103	103	106	100	13	0
16	104	104	112	112	48	0
24	—	—	—	—	152	0
48	—	—	—	—	—	0

* The test organism was a strain of *Streptococcus faecalis* var. *zymogenes* and growth was measured as optical density × 100 at 640 mμ.

some CO_2 was absorbed into the medium during the sterilization by Seitz filtration, but the addition of a small amount of carbonate was found to enhance growth in this medium as well as in tryptone-yeast-glucose broth. Superior growth was also obtained when the fitration step was omitted, both in the presence or absence of added carbonate.

Glycine inhibition. Under alkaline conditions, glycine has been found to be relatively toxic to the enterococci. Table 2 illustrates the effect of 0.05 M glycine upon the growth of *S. faecalis* var. *zymogenes* at different pH levels. The other strains and species studied exhibited similar behavior. The effect was slight at pH 7.0 and 8.3 but quite marked at pH 9.6. Figure 1 illustrates further the sharp rise in toxicity of 0.01 and 0.05 M glycine for *S. faecalis* var. *zymogenes* at pH 10.0. It has also been found that as little as 0.006 M glycine (the amount used to buffer the medium of Shattock and Hirsch) caused some inhibition at pH 9.6. Thus, Skadhauge (1950), who used a carbonate buffer, would be expected to have obtained superior results in determining the ability of enterococci to grow at pH 9.6 as compared to investigators using a medium buffered with glycine.

Inhibition by glycine peptides and D-amino acids. The growth response of the enterococci at pH 10.0 to glycine in the free form and as a

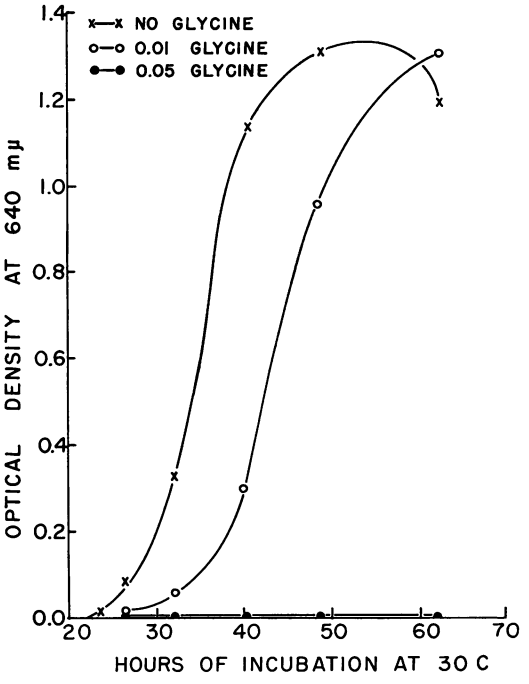

Figure 1. Toxicity of glycine for a typical strain of enterococcus under alkaline conditions. Growth of *Streptococcus faecalis* var. *zymogenes* in pH 10.0 tryptone-yeast-glucose broth.

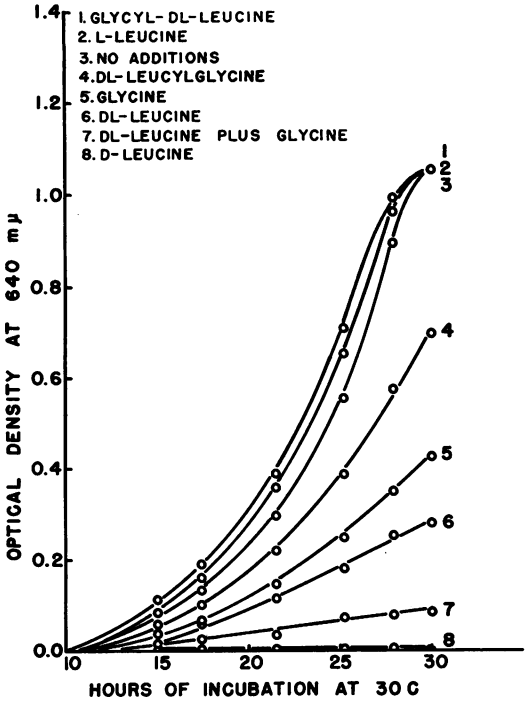

Figure 2. Effect of glycine, leucine stereoisomers, and their peptide combinations at 0.02 M concentration on the growth of *Streptococcus faecalis* var. *zymogenes* at pH 10.0.

TABLE 3

*Effect of amino acid stereoisomers upon the growth of an enterococcus in alkaline media**

Amino Acid Added to Tryptone Yeast-Glucose Broth	Optical Density × 100	
	L-Isomer	D-Isomer
None	27	27
Aspartic acid	130	42
Glutamic acid	140	60
Histidine	90	1
Isoleucine	130	12
Leucine	116	9
Methionine	130	0
Phenylalanine	32	0
Serine	100	2
Tryptophan	100	1
Valine	130	15

* The test organism was a strain of *Streptococcus faecalis* var. *zymogenes*. The media were adjusted to pH 9.9 and uninoculated control tubes were shown to retain this pH level throughout the experiment. Growth was measured after 48 hr at 30 C.

dipeptide was studied. Amino acids and peptides were autoclaved dry for 5 min and then taken up in 4× concentration in dilute, sterile NaOH. The growth behavior of *S. faecalis* var. *zymogenes* at pH 10.0 in the presence of 0.02 M concentrations of leucine isomers and glycine, free and as a dipeptide, is shown in figure 2.

Certain features of the response of *S. faecalis* var. *zymogenes* to these stereoisomers and dipeptides were exhibited by all the enterococci tested. The inhibition caused by DL-leucine was attributable to the D-form in all cases; at the concentration used the L-form was stimulatory. Glycyl-DL-leucine was either more stimulatory or less toxic than DL-leucylglycine. Both peptides always were either less toxic or more stimulatory than an equivalent amount of DL-leucine plus glycine. A similar pattern of results was observed with DL-alanylglycine and glycyl-DL-alanine with the exception that DL-alanylglycine had a more favorable growth effect. Thus, in the case of the glycine-leucine peptides, the growth response was more favorable when the asymmetrical center of the molecule was in C-terminal residue

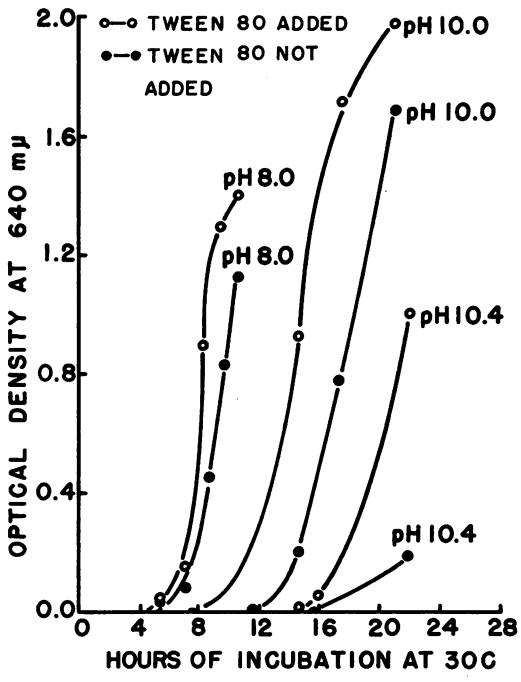

Figure 3. Effect of 0.05 per cent Tween 80 on the growth of a strain of *Streptococcus faecalis* var. *liquefaciens* in alkaline media.

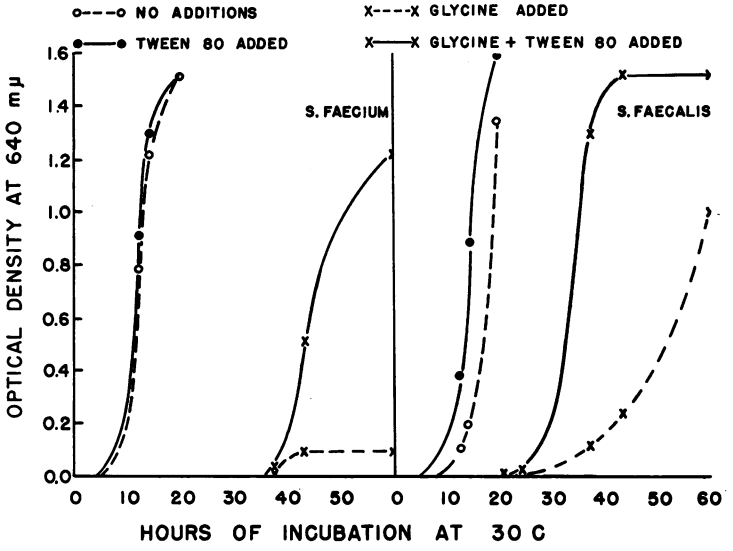

Figure 4. Effect of 0.05 per cent Tween 80 on the toxicity of 0.05 M glycine for enterococci at pH 9.6.

while this relationship was reversed with the glycine-alanine peptides.

A further comparison of the toxicity of D- and L-isomers at pH 9.9 is presented in table 3. It is apparent that the D-isomer was either toxic or less stimulatory in all cases. In the specific experiment cited in this table growth in the unsupplemented medium was much poorer than usual, thus emphasizing the stimulatory effect of the L-isomers other than phenylalanine.

Oleate stimulation. Tween 80 (polyoxyethylene sorbitan monooleate) was found to stimulate growth of enterococci at higher pH levels (fig 3). Furthermore, it reduced the toxicity of added glycine. Figure 4 illustrates these effects on typical enterococcus strains. Similar results were observed with Span 80 (sorbitan monooleate). Other Tweens and Spans showed diminished ability or inability to produce these effects.

Media for demonstrating alkali tolerance of the enterococci. As indicated by the preceding results, a very satisfactory medium for growing enterococci in an alkaline environment is tryptone-yeast-glucose broth containing 0.05 M carbonate and 0.05 per cent Tween 80, or Span 80. The basal medium, less K_2HPO_4 and Na_2CO_3, but containing the surfactant, is autoclaved 10 to 12 min at 2× concentration (longer autoclaving produces a less satisfactory medium). The buffer salts are autoclaved together at 4× concentration in screw cap bottles. After mixing, the pH is adjusted with sterile 2 N NaOH and 2 N H_3PO_4 and the concentration brought to single strength with sterile distilled water. The medium is dispensed into sterile screw cap tubes in 10.0-ml amounts. With this medium it was found that all enterococci tested could initiate growth up to pH 10.5.

TABLE 4

Comparative efficiency of pH 10.0 broth and azide-dextrose broth as an enrichment medium for enterococci from feces

Enrichment Broth	Enterococci (× 10^6) per Ml of Enrichment Broth*			
	Avian Feces	Human Feces —1	Human Feces —2	Human Feces —3
Azide-dextrose	0.9	66	0.2	None
Tryptone-yeast-glucose + Tween 80 + carbonate at pH 10.0	1700	1300	70	1200

* This represents the quantitative count on the enterococcus confirmatory medium.

To test the usefulness of the medium as a selective enrichment broth for enterococci, tubes adjusted to pH 10.0 were inoculated with approximately equal quantities of one avian and three human fecal samples. Similar amounts of these specimens also were inoculated into azide-

dextrose broth (Difco). After 18 hr at 30 C, the material in the enrichment tubes was plated on enterococcus confirmatory agar (Difco). The results obtained with the four fecal samples are summarized in table 4. The tryptone-yeast-glucose broth containing carbonate and Tween 80 was superior to the azide-dextrose broth for each sample.

Twenty strains of bacteria were isolated from the pH 10.0 broth inoculated with fecal samples by plating on APT agar (Case) and all proved to be *S. faecium*. Fifteen strains were isolated from the azide-dextrose broth, only 5 of which proved to be enterococci (4 were *S. faecalis* types and 1 was *S. faecium*). Thus, the pH 10.0 broth appeared to be a superior enterococcus enrichment broth, but the results suggest that either this medium favored the growth of *S. faecium* or the azide-dextrose broth favored the growth of *S. faecalis*. These results demonstrate once again the difficulty of using an enrichment broth to determine the flora in a given environment. Consequently, it would appear that this medium can be used at pH 10.0 as an enrichment broth, or as a taxonomic test to detect alkali tolerance of the enterococci. In the latter case it might be preferred to adjust the medium to pH 9.6 in accordance with the criterion as generally stated.

SUMMARY

Growth of enterococci in media having a high initial pH is stimulated markedly by carbonate and to a lesser extent by oleate. Growth in these media is inhibited by glycine and by the D-isomer of a variety of amino acids. Peptides of glycine are less inhibitory than the free acid. In a favorable medium, buffered with carbonate rather than glycine, all enterococci tested initiated growth up to pH 10.5. Such a medium adjusted to pH 10.0 has been shown to be a superior enrichment broth for the detection of enterococci in fecal samples and presumably from other sources. It also provides a convenient taxonomic tool for differentiating enterococci from other streptococci.

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