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Effect of Buffering Media with Phosphates on Antibiotic Resistance of Lactic Streptococci†

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Buffering complex growth media with inorganic or organic phosphates increased the resistance levels of lactic streptococci to aminoglycoside antibiotics, whereas it increased their sensitivity to erythromycin, penicillin, and tetracycline. The results suggest the need for caution in the selection of media for testing the antibiotic sensitivity of lactic streptococci.

The antibiotic resistance of lactic acid bacterial cultures has been tested in many laboratories, and information on the levels of various antibiotics causing partial or complete inhibition has been compiled (2, 4, 8, 9). Conflicting results for the sensitivity of the cultures to different antibiotics have been reported (9, 12, 13). Although the reasons for such an anomaly have been unknown, strains of bacteria showing resistance to drugs have not been recommended for industrial use (13).

During the isolation from lactic streptococci of antibiotic-resistant strains to be used in genetic recombination experiments, it was noticed that a strain was sensitive to streptomycin (Sm), neomycin (Nm), and kanamycin (Km) when assayed on lactic agar (3) plates, whereas it was highly resistant to these drugs on M17 (14) agar plates. A critical examination of the two media indicated that the striking difference between them is the presence of organic phosphate in M17, besides higher levels of peptides and proteins. Since M17 broth is extensively used for bacterial growth (7) and for genetic recombination experiments with lactic streptococci (5, 6, 10), the present investigation was initiated to examine the effect of buffering media with both organic and inorganic phosphates on the antibiotic resistance levels of several cheese and yogurt starter cultures.

The following bacterial strains were used: *Streptococcus lactis* C2, SL712, ML3, and SK1; *Streptococcus cremoris* AM1, ML1, and SC607; *Streptococcus thermophilus* X1, Y1, and Z1. In addition to the above pure cultures, one independent tetracycline (Tc)-resistant mutant isolated spontaneously in this laboratory from M17 plates containing 2 μg of Tc per ml was also used. The mutant (Z1-8) showed resistance to 8 μg of Tc per ml, whereas the parental strain was only resistant to 1 μg of Tc per ml. All the strains used were from the culture collection of the Food Research Institute.

The media used were M17 and modified M17, referred to here as M17$^-$ without β-glycerophosphate (GP) and M17P in which GP was replaced by inorganic phosphates (K_2HPO_4 [1.33%] + KH_2PO_4 [0.57%][wt/vol]). The pH of each broth was adjusted to 7.1 ± 0.05 before autoclaving (15 min, 15 lb of pressure at 121°C). In some experiments, lactic broth with and without 1.9% inorganic or organic phosphates was also used for comparisons. The plates were made by adding 1.5% (wt/vol) agar to broth before autoclaving.

The antibiotics used were as follows: penicillin G sodium (Pen) and tetracycline hydrochloride (Tc) from Nutritional Biochemicals Corp., Cleveland, Ohio; streptomycin sulfate (Sm), neomycin sulfate (Nm), kanamycin sulfate (Km), and erythromycin (Em) from Sigma Chemical Co., St. Louis, Mo. All antibiotic solutions were prepared in concentrated aqueous solutions, filter sterilized, and kept frozen at −20°C until used.

All the bacterial strains were grown in M17 broth. Cultures of *S. lactis* and *S. cremoris* were routinely grown at 32°C from a 2% inoculum of an overnight culture, and the cultures of *S. thermophilus* were grown at 37°C. The overnight culture (0.1 ml) of each strain was surface spread on M17, M17$^-$, and M17P agar plates or lactic agar (LA) plates containing the desired concentrations of antibiotics to be tested. The plates with *S. thermophilus* were incubated at 37°C in anaerobic jars (BBL Microbiology Systems, Cockeysville, Md.), and the plates with *S. lactis* and *S. cremoris* were incubated at 32°C for 48 h.

Several strains of cheese and yogurt starter cultures were tested for their resistance to Nm, Sm, and Km on M17, M17$^-$, and M17P plates. All the cultures showing sensitivity to Nm, Sm, and Km on M17$^-$ plates exhibited increased levels of resistance on M17 and M17P plates (Table 1). This indicates that phosphate in the medium inhibits the antibacterial activity of the antibiotics. A comparative examination of the effects of the two kinds of phosphates showed that the bacterial strains in general were more resistant to high levels of all three antibiotics when assayed on the medium containing inorganic phosphate (Table 1). Further comparisons among the cultures of *S. cremoris*, *S. lactis*, and *S. thermophilus* revealed that the *S. cremoris* strains were the least resistant.

All three drugs tested belong to the main aminoglycoside antibiotics and have very similar chemical structures as well as similar antibacterial spectra (1, 11). Therefore, similar results were expected. Thus, it became of interest to test the action of phosphates on antibiotics belonging to other groups.

All the strains were then tested for resistance to Tc, Pen, and Em antibiotics on M17$^-$, M17, and M17P plates. The strains exhibiting resistance to all three drugs on M17$^-$ agar plates showed sensitivity on M17 and M17P plates (Table 2). The sensitivity was more pronounced in the presence of inorganic phosphate than with organic phosphate. The results were similar in all the strains tested and were in contrast to those obtained with aminoglycoside antibiotics (Table 1).

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TABLE 1. Effect of inorganic and organic phosphates on Sm, Nm, and Km resistance of several lactic acid bacterial cultures[a]

Medium	Antibiotic	Concn (μg/ml)	*S. lactis*				*S. cremoris*			*S. thermophilus*			
			C2	SL712	ML3	SK1	AM1	ML1	SC607	X1	Y1	Z1	Z1-8
M17⁻	Sm	50	R	R	R	R	S	S	S	R	R	R	R
M17	Sm		R	R	R	R	R	S	S	R	R	R	R
M17P	Sm		R	R	R	R	R	R	R	R	R	R	R
M17⁻	Sm	100	S	S	S	S	S	S	S	S	S	S	S
M17	Sm		R	R	R	R	S	S	S	S	R	R	R
M17P	Sm		R	R	R	R	S	S	R	R	R	R	R
M17⁻	Sm	200	S	S	S	S	S	S	S	S	S	S	S
M17	Sm		R±	S	R±	S	S	S	S	S	S	S	S
M17P	Sm		R	R	R	R	S	S	S	R	R	R	R
M17⁻	Nm	1,000	S	S	S	S	S	S	S	S	S	S	S
M17	Nm		S	S	R	S	R	S	S	S	R	R	R
M17P	Nm		R	R	R	R	R	R	R	R	R	R	R
M17⁻	Km	100	S	S	S	S	S	S	S	S	S	S	S
M17	Km		R	R±	R	R	S	S	S	R	R	R	R
M17P	Km		R	R	R	R	R	R	R	R	R	R	R
M17⁻	Km	200	S	S	S	S	S	S	S	S	S	S	S
M17	Km		S	S	R±	S	S	S	S	R±	R	R	R
M17P	Km		R	R	R	R	S	S	R±	R	R	R	R
M17⁻	Km	400	S	S	S	S	S	S	S	S	S	S	S
M17	Km		S	S	S	S	S	S	S	S	S	R	R
M17P	Km		R	R	R	R	S	S	S	R	R	R	R

[a] Each culture was grown overnight at 32°C in M17 broth, and 0.1 ml containing 1×10^8 to 5×10^8 CFU was surface spread on each plate. Plates were incubated for 48 h at 32°C, and then colonies were counted. A culture with 0 to 50 colonies per plate was considered sensitive (S), a culture with 51 to 100 colonies per plate was considered partially resistant (R±), and a culture with >100 colonies per plate was considered resistant (R).

TABLE 2. Effect of inorganic and organic phosphates on Pen, Tc, and Em resistance of several lactic acid bacterial cultures[a]

Medium	Antibiotic	Concn (μg/ml)	*S. lactis*				*S. cremoris*			*S. thermophilus*			
			C2	SL712	ML3	SK1	AM1	ML1	SC607	X1	Y1	Z1	Z1-8
M17⁻	Pen	0.2	R	R	R	R	S	R	R	S	S	S	S
M17	Pen		R	R	R	R	S	R	R	S	S	S	S
M17P	Pen		S	S	S	S	S	S	S	S	S	S	S
M17⁻	Pen	0.4	R	R	R	R	S	S	S	NT	NT	NT	NT
M17	Pen		S	S	R	S	S	S	S	NT	NT	NT	NT
M17P	Pen		S	S	S	S	S	S	S	NT	NT	NT	NT
M17⁻	Em	0.2	R	R	R	R	R	R	R	R	R	R	R
M17	Em		R	R	R	R	S	S	R	R	R	R	R
M17P	Em		S	R	R	R	S	S	S	S	S	S	S
M17⁻	Em	0.4	R	R	R	R	S	S	R	NT	NT	NT	NT
M17	Em		S	R	S	S	S	S	S	NT	NT	NT	NT
M17P	Em		S	S	S	S	S	S	S	NT	NT	NT	NT
M17⁻	Tc	1.0	R	R	R	S	R±	S	S	R±	R	R	R
M17	Tc		R	R	R	S	S	S	S	R	R	R	R
M17P	Tc		S	S	S	S	S	S	S	S	S	S	R
M17⁻	Tc	8.0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	S
M17	Tc		NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	R
M17P	Tc		NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	S

[a] Sensitivity was tested as described in Table 1, footnote *a*. For an explanation of symbols, see Table 1, footnote *a*. NT, Not tested.

TABLE 3. Effect of the incorporation of inorganic and organic phosphates in LA on Km (100 μg/ml) and Tc (1 μg/ml) resistance of lactic streptococci

Medium[a]	Antibiotic	*S. lactis*				*S. cremoris*			*S. thermophilus*
		C2	ML3	SK1	SL712	AM1	ML1	SC607	Z1
LA	Km	R±	S	S	S	S	S	S	R±
LA + GP	Km	R	R	R	R	S	S	R±	R
LA + P	Km	R	R	R	R	R	R	R	R
LA	Tc	R	R	S	R	R	S	S	R
LA + GP	Tc	R	R	S	R	S	S	S	R
LA + P	Tc	S	S	S	S	S	S	S	S

[a] LA with desired concentrations of antibiotics was prepared. LA + GP and LA + P contained 1.9% GP and inorganic phosphates (P), respectively, as described in the text. Sensitivity was tested as described in Table 1, footnote *a*.

To obtain more information on whether the phosphate affects only the intrinsic resistance levels of normal bacteria, a Tc-resistant mutant of *S. thermophilus* Z1 was also included in the testing. This mutant also became sensitive to Tc in the presence of inorganic phosphate (Table 2). Thus, all six antibiotics can be divided into two groups, based on their action when tested in the presence of phosphate. The aminoglycoside antibiotics (Nm, Sm, and Km), being inhibited in their antibacterial activity, belong to one group, whereas Tc, Pen, and Em, exhibiting increased antibacterial activity, belong to another group.

The antibiotic sensitivity of the bacterial strains on the LA plates was compared with that obtained on $M17^-$ agar plates. The effect of the incorporation of phosphates in lactic broth was also compared with the effect in M17 and M17P media. For this comparison, Km and Tc, representative of the two groups of antibiotics, were selected for testing.

All the cultures tested showed similar resistance levels to the antibiotics on LA and $M17^-$ plates (Tables 1, 2, and 3). Again, the cultures tested showed resistance to Km and sensitivity to Tc on LA plates containing phosphates, whereas they showed sensitivity to Km and resistance to Tc on the plates without any added phosphates (Table 3). Thus, it became clear that the increased levels of peptides and proteins in M17 medium were not responsible for the variations in the antibiotic resistance levels observed. At the same time, it also became clear that buffering of lactic broth with phosphates had effects on antibiotics similar to those observed in buffering of M17 and M17P media with phosphates.

The results of the present investigation suggest that phosphates in the growth media affect a pathway of action common to all the antibiotics tested. The phosphates interfered with the action of aminoglycoside antibiotics and increased the lethal concentrations of the drugs. On the other hand, they accelerated the action of Tc, Pen, and Em, thus decreasing the concentrations of the drugs required for lethality.

In the manufacture of cheese with mixed starters, the antibiotic residues in the milk should be less of a problem for *S. lactis* strains, since they are fairly resistant as compared with *S. cremoris* strains. However, the balance between the *S. lactis* and *S. cremoris* strains could be upset, resulting in a low-quality product. The results thus suggest the need for caution in the selection of media for testing the antibiotic resistance of cheese and yogurt starter cultures.

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