STUDIES OF STAPHYLOCOCCI WITH SPECIAL REFERENCE TO THE COAGULASE-POSITIVE TYPES[1]

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Studies of staphylococci have centered largely around attempts to establish simple laboratory tests that could be used as criteria for the differentiation of pathogenic from nonpathogenic strains. The term "pathogenic," when applied to staphylococci, includes any strain capable, under optimum conditions, of causing an infection or causing a food poisoning.

The best indicator of pathogenic potentiality has been the ability to coagulate oxalated or citrated blood plasma. Other characteristics of some importance are the fermentation of mannitol, orange pigmentation, and the ability to grow in the presence of 7.5 per cent sodium chloride. However, a number of apparently nonpathogenic staphylococci (coagulase-negative) are positive on some of the latter tests.

Evans (1947) has suggested that the anaerobic fermentation of mannitol may be of value for such studies. The introduction of media containing 7.5 per cent sodium chloride by Chapman (1945, 1946*a*, 1946*b*) has greatly simplified selective plating for staphylococci. The high salt concentration greatly enhances pigment production by the orange strains, and it inhibits most contaminants with the exception of some sporeforming bacilli.

Nutritional studies of staphylococci have been confined largely to studies on known or supposed pathogens, although in some cases pathogenicity was either not mentioned or not adequately established. Knight (1937) showed that thiamine and nicotinic acid were essential for *Staphylococcus aureus*. Porter and Pelczar (1941) found two mucoid, orange strains from clinical sources that required biotin. Sevag and Green (1944) reported two strains of "toxigenic" staphylococci that required pantothenic acid in the absence of tryptophan, and Vilter and Spies (1940) noted that pyridoxine stimulated growth of a white *Staphylococcus*.

In the present work an attempt has been made to correlate various physiological tests and the nutritional requirements of a collection of staphylococci. The coagulase test has been used as the criterion of pathogenicity. The 19 coagulase-positive strains were from clinical infections, food poisonings, and frozen foods. The 66 coagulase-negative strains were all from frozen foods.

PHYSIOLOGICAL STUDIES

Methods. All cultures were carried in beef infusion broth at 35 C. All inoculations were made from approximately 24-hour cultures. In critical tests, such as the coagulase test and mannitol fermentation, the cultures were transferred

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daily for at least 5 days prior to inoculation of the test media. This was particularly important for old stock cultures.

Difco coagulase plasma was used for the coagulase test. A suspension of 0.5 g of the dried plasma was prepared in 15 ml of distilled water. To 0.5 ml of this suspension, in a small tube, were added 2 drops of a 24-hour culture from beef infusion broth. The tube was shaken to mix the culture and plasma and then placed in an incubator at 35 C. Any degree of coagulation within 24 hours was considered positive.

Mannitol fermentation was tested on a medium containing 0.3 per cent beef extract, 0.5 per cent tryptone, 0.5 per cent mannitol, and 0.004 per cent bromcresol purple. For anaerobic fermentation tests, the medium was heated in flowing steam for 15 minutes to drive out dissolved air, and cooled immediately before inoculation. Following inoculation, each tube was sealed with melted "vaspar." Tubes were incubated at 30 C for 10 days. Daily observations were made for changes in the color of the bromcresol purple. Final pH was determined using a Beckman potentiometer. The fermentation of glucose, lactose, sucrose, and glycerol was tested in the same manner.

Pigmentation was determined by inoculating cultures on beef infusion agar slopes and on slopes of Chapman's medium no. 110 (Chapman, 1946*a*). For cultures that would grow in the presence of 7.5 per cent NaCl, medium no. 110 gave much stronger pigmentation and showed pigment with some cultures that were white on the beef infusion agar slopes.

Stone's extract gelatin agar was inoculated, incubated for 48 hours at 35 C, and tested in the usual manner. Nutrient gelatin contained 0.3 per cent beef extract, 0.5 per cent tryptone, and 4.0 per cent gelatin. It was inoculated with 1 drop of culture, incubated for 10 days at 30 C, and placed in an ice water bath until a control tube was thoroughly solidified.

The phenol red mannitol salt agar was a Difco dehydrated medium containing 7.5 per cent salt, following the suggestion of Chapman (1945). Cultures were streaked on it, and any yellow zone appearing within 48 hours at 35 C was considered positive. The bromcresol purple mannitol salt agar contained 0.3 per cent beef extract, 0.5 per cent tryptone, 7.5 per cent NaCl, 1.0 per cent mannitol, 1.5 per cent agar, and 0.004 per cent bromcresol purple. Cultures were tested by the method used with the phenol red medium.

Results. The results of the physiological tests are summarized in table 1. The ability to ferment mannitol under anaerobic conditions (Evans, 1947) was possessed by all the coagulase-positive strains, and by only 2 of the 47 coagulase-negative strains that fermented mannitol under the ordinary conditions. These two strains (S-36 and S-37) were isolated from the same plate and were identical in all tests, hence were probably duplicates. They produced an orange pigment, but differed in many other respects from the coagulase-positive cultures. They were negative on the Stone test, failed to grow in the presence of 7.5 per cent salt, and required added thiamine, nicotinic acid, biotin, pyridoxine, and pantothenic acid.

As noted previously (Evans, 1947), the coagulase-positive strains tended to produce only a small amount of acid when grown on the surface of mannitol agar

containing 7.5 per cent NaCl. With bromcresol purple as the indicator, the coagulase-positive strains produced very little or no yellow zone. This may be

TABLE 1

Summary of physiological tests

	PER CENT POSITIVE		
	Coagulase +	Coagulase—	
		Mannitol +	Mannitol—
Mannitol fermentation			
Aerobic	100	100	0
Anaerobic	100	4	0
Pigmentation			
Orange	89	17	16
Cream	11	9	0
Yellow	0	0	16
Gelatin reactions			
Stone's gelatin	89	6	68
Nutrient gelatin	79	15	74
P.R. mannitol salt agar	100	96	0
B.C.P. mannitol salt agar	0	85	0
Glucose fermentation			
Aerobic	100	100	95
Anaerobic*	100	90	86
Lactose fermentation	100	64	42
Sucrose fermentation	100	98	74
Glycerol fermentation†			
Aerobic	100	100	—
Anaerobic	0	0	—
Litmus milk reactions			
Acid production	89	19	32
Acid curd	68	2	32
Proteolysis	0	4	16

* Only 39 strains were tested (11 coagulase-positive; 21 coagulase-negative, mannitol-positive; and 7 coagulase-negative, mannitol-negative).

† Only 18 strains were tested (11 coagulase-positive; and 7 coagulase-negative, mannitol-positive).

due to a more efficient aerobic respiration mechanism, or a greater Pasteur effect. When the 7.5 per cent NaCl was omitted from the medium, more acid was produced and was indicated by bromcresol purple.

NUTRITIONAL STUDIES

Methods. Vitamin requirements were determined by omitting the vitamins one at a time from a complete medium composed of the basal medium and the seven B vitamins given in table 2. Enough medium was prepared in one batch to allow for a serial transfer after 24 hours. The medium, which was not used for 24 hours, was steamed for 15 minutes and cooled before inoculation. All tubes were incubated at 30 C. The amount of growth was determined by measuring turbidity with an Evelyn type colorimeter designed for use as a densitometer. The circuits were so arranged that complete light transmission (through a

TABLE 2

Medium for nutritional studies

BASAL MEDIUM	WEIGHT PER 100 ML OF MEDIUM
	mg
Casein hydrolyzate	50
DL-Tryptophan	1
L-Cystine	1
Glucose	100
K_2HPO_4	40
Salts	
$MgSO_4 \cdot 7H_2O$	2
NaCl	0.10
$FeSo_4 \cdot 7H_2O$	0.10
$MnSO_4 \cdot 4H_2O$	0.10
Adenine·SO_4	0.05
Guanine·HCl	0.05
Uracil	0.05
Xanthine	0.05
VITAMIN SOLUTIONS	
Thiamine·HCl	0.01 mg
Nicotinic acid	0.05 "
Biotin	0.01 μg
Riboflavin	0.01 mg
Pyridoxine·HCl	0.01 "
Pantothenic acid (Ca)	0.01 "
Folic acid	0.10 μg

16.0-mm tube of distilled water) gave a reading of zero, and no light transmission gave a reading of 100 on the galvanometer. Permanently installed in the instrument was a red filter transmitting a band of light that centered at about 660 A. With bacterial cultures, readings from 20 to 90 were approximately linear. Tubes reading below 10 generally showed no turbidity to the naked eye, and tubes of sterile media generally gave readings around 5. The stock solutions were assayed for any contaminating vitamins using streptococci of known vitamin requirements.

Results. The complete vitamin requirements were determined for 40 strains.

TABLE 3

Vitamin requirements

	COAGULASE-POSITIVE	COAGULASE-NEGATIVE	
		Mannitol+	Mannitol—
Strains tested	19	18	3
Require thiamine	19	18*	2
" nicotinic acid	19	16	3
" biotin	0	18	3‡
" riboflavin	0	0	0
" pyridoxine	0	2†	0
" pantothenic acid	0	6	1
" folic acid	0	0	0

* Three strains grew to about half-maximum without thiamine.

† These were the same two strains that fermented mannitol anaerobically.

‡ Two strains grew to about half-maximum without biotin.

TABLE 4

Biotin requirement

STRAIN	DENSITOMETER READINGS							
	Complete medium				Minus biotin			
	1st transfer		2nd		1st		2nd	
	24 hr	48	24	48	24	48	24	48
Coagulase +								
S-1	60	63	64	68	32	42	26	61
S-6	72	80	63	75	66	77	53	73
S-14	66	68	68	70	40	67	30	61
209	67	72	66	70	51	56	44	56
64	47	52	27	53	26	37	24	50
99	53	68	60	69	27	55	37	58
161	37	55	36	56	17	45	23	43
177	56	62	58	64	27	52	40	52
185	48	58	56	67	33	57	47	56
197	33	54	47	59	20	45	30	50
Coagulase — Mannitol +								
75	56	72	56	69	15	27	6	4
S-18	55	61	54	64	6	16	9	5
S-19	36	61	38	64	10	29	9	19
S-37	31	63	10	65	3	10	5	7
13–12	30	61	55	61	8	15	10	14
13–20	59	75	54	71	8	9	6	4
15–1	52	55	50	58	10	15	7	4
Coagulase — Mannitol —								
8–10	6	48	9	50	6	6	6	6
20–1	32	71	54	72	8	45	6	36
22–7	26	76	43	78	7	38	5	23

The complete medium contained the usual base plus the 7 vitamins. The other medium was identical except for biotin.

The results are summarized in table 3. It will be noted that the ability to grow well in the absence of added biotin was limited to the coagulase-positive strains.

An additional experiment covering the biotin requirement of the 19 coagulase-positive strains and 29 of the coagulase-negative strains gave essentially the same results. Turbidity readings for 20 of these cultures are given in table 4. The cultures for which data are not given gave very similar readings. Biotin exerted a mild stimulatory effect on some of the coagulase-positive strains, particularly in the first 24 hours of growth. Also, some of the coagulase-negative strains were able to grow slightly without added biotin, but a wide difference in growth response between the two groups is still obvious. This was even more striking when the strains unable to ferment mannitol were eliminated. Since Porter and Pelczar (1941) did not give any physiological data on their two strains

TABLE 5

Effect of omissions from the basal medium

	COMPLETE	MINUS TRYPTOPHAN	MINUS CYSTINE	MINUS PURINES AND PYRIMIDINES
Coagulase +				
209	69	68	34	61
64	58	53	28	52
99	71	68	31	68
S-6	79	75	50	74
S-14	69	68	36	59
177	75	61	35	65
197	61	61	32	43
Coagulase −				
75	69	67	65	67
S-18	58	59	52	53
13–20	73	36	56	68

Densitometer readings of 10 mannitol-fermenting strains (second transfer, 48 hours) showing the effect of omitting components from a complete medium (basal plus thiamine, nicotinic acid, and biotin).

which required biotin, it is possible that they may have been coagulase-negative.

The effect of omitting some components of the basal medium is shown in table 5. There was no appreciable effect when tryptophan was omitted except on one coagulase-negative strain, which achieved only about half-maximum growth. Cystine appeared to be definitely stimulatory to the coagulase-positive strains, under the conditions used. The omission of adenine, guanine, uracil, and xanthine had little or no effect on the growth of the 10 strains tested. All of this would be expected in view of the work of Surgalla and Hite (1946) and Surgalla (1947), who grew enterotoxic strains in a medium containing only arginine, cystine, glucose, salts, nicotinic acid, and thiamine.

DISCUSSION

Although the strains included in this study are admittedly few in number, the results indicate that the staphylococci that give a positive coagulase reaction

comprise a rather homogenous group. They ferment mannitol, tend to produce pigment, generally give a positive Stone reaction, ferment glucose, lactose, sucrose, and glycerol, and require added thiamine and nicotinic acid. All of the foregoing characteristics have been emphasized by previous investigators, but there are many coagulase-negative strains having several or all of these characteristics. They may be separated, however, by the ability of the coagulase-positive strains to ferment mannitol under anaerobic conditions and to grow in a synthetic medium devoid of biotin. These results appear to be contradictory to those of Colwell (1939), who reported 23 orange-pigmented strains from clinical sources to be unable to ferment mannitol anaerobically. However, the coagulase test was not run on those cultures and their pathogenicity was not clearly established. Also, it should be pointed out that active cultures are essential to this test, and stock cultures should be serially transferred for several days before testing.

The pantothenic acid requirement of 7 coagulase-negative strains was shown in the presence of tryptophan. No attempt was made in the present work to establish a pantothenic acid requirement in the absence of tryptophan, as was demonstrated by Sevag and Green (1944). However, in the experiment reported in table 5, when tryptophan was omitted from a medium that contained no pantothenic acid, only one culture (coagulase-negative) showed any significant decrease in growth. Other tests had shown this batch of casein hydrolyzate to be free of tryptophan.

The requirement for pyridoxine shown by strains S-36 and S-37 was unique among the cultures studied. Vilter and Spies (1940) reported that pyridoxine was stimulatory for one strain of *Staphylococcus albus*, but the present report seems to be the first record of pyridoxine being essential for a *Staphylococcus*.

The phenol red mannitol salt agar (Chapman, 1945) proved to be a satisfactory selective plating medium, although it was observed that some sporeforming bacilli were able to grow on this medium when it was used for checking frozen foods. Also, a number of coagulase-negative staphylococci grew on the medium and produced acid. No experiments have been directed toward incubating these plates under anaerobic conditions, although this technique might prove to be superior for both differential and selective purposes.

SUMMARY

The 19 coagulase-positive staphylococci studied comprised a rather homogenous group. They could be separated from the 66 coagulase-negative strains studied by their ability to ferment mannitol under anaerobic conditions, and their ability to grow in a synthetic medium devoid of biotin.

Among the coagulase-negative staphylococci, 6 strains were found to require pantothenic acid and 2 required pyridoxine. These requirements appear to be unique among the staphylococci thus far reported.

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