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Frequency of staphylococcal lysozyme production tested by plate method[1]

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SYNOPSIS Lysozyme production is a frequent property of staphylococcal strains isolated from various sources; all 503 tested strains of *Staphylococcus aureus* and 13 out of 35 strains of *Staphylococcus epidermidis* produced an enzyme lysing *Micrococcus lysodeikticus* as tested by a modified plate method. Lysozyme production by staphylococci is more frequent than the production of free coagulase, clumping factor, staphylokinase, Tween 80 lipase, and $HgCl_2$ resistance.

The staphylococcal lysozyme produced by *Staphylococcus aureus* strain 524 is an enzyme by which living cells and the cell walls of *Micrococcus lysodeikticus* are lysed. Its action results in the appearance of degradation products of the cell walls, which react as N-acetylamino sugars and contain free reducing groups (Richmond, 1959). The staphylococcal lysozyme also acts on murein isolated from cell walls of the producing strain, as well as on cell walls of *Staphylococcus epidermidis.* A number of properties of staphylococcal lysozyme have been determined (Hawiger, 1968) and compared with those of crystalline egg white lysozyme (E.C. 3.2.1.17 mucopeptide N-acetylmuramyl hydrolase). The two enzymes exhibit some similarities.

It seemed to be of interest to determine by uniform methods the incidence of lysozyme production by staphylococci isolated mainly from pathological cases. For comparative purposes the determination included also the production of some agents important in the differentiation of staphylococci recently isolated in epidemiological work (Jevons, John, and Parker, 1966). On the other hand, for practical reasons it was decided to evaluate the possibility of lysozyme determination by the plate method in the routine diagnosis of staphylococci.

MATERIAL AND METHODS

STAPHYLOCOCCAL STRAINS The following strains were tested: 143 strains obtained from the territorial sanitary epidemiological stations, where they were isolated from current diagnostic specimens collected from patients; 175 strains isolated from burnt patients admitted to hospital in the Clinic of Surgery, Institute of Haematology, Warsaw, were obtained by courtesy of Dr. Serafińska and Dr. Zak; 182 strains from the collection of this laboratory, including standard strains for phages, standard strains with stable antigenic properties used for preparing immune sera (Cowan I, II, III, Oeding's strains); classic strains used for isolating individual staphylococcal toxins and enzymes (Newman, Wood 46, V-8, E_7, 524); enterotoxic strains, strains of animal origin; strain 502 A; and 43 strains isolated in routine diagnostic work in this laboratory.

IDENTIFICATION OF STRAINS All the strains of Gram-positive cocci were identified according to the principles and methods recommended in Cowan's and Steel's manual (Cowan and Steel, 1965). Catalase, glucose oxidation, and fermentation (OF test), mannitol fermentation, and the production of free coagulase and clumping factor were determined.

The properties of the strains were determined as follows: staphylokinase production was determined by the plate method (Lack and Wailling, 1955); the production of Tween 80-splitting lipase was tested by the plate method (Sierra, 1952); $HgCl_2$ resistance was determined by the plate method (Moore, 1960).

DETERMINATION OF STAPHYLOCOCCAL LYSOZYME PRODUCTION BY THE MODIFIED PLATE METHOD A suspension of *M. lysodeikticus* cells killed by heat is present in the medium. After solidification of the medium, which becomes opaque, staphylococcal strains are inoculated on it. During incubation at 37°C zones of transparency appear around the growing colonies, due to lysis of *M. lysodeikticus* cells by lysozyme produced by the growing strain.

Medium The medium for determining lysozyme production is that of Goldbach and Haenel (1964). Take 0·4% yeast extract, 0·6% glucose, and 0·1% Tween 80 made up to 100% with nutrient agar (pH = 6·8).

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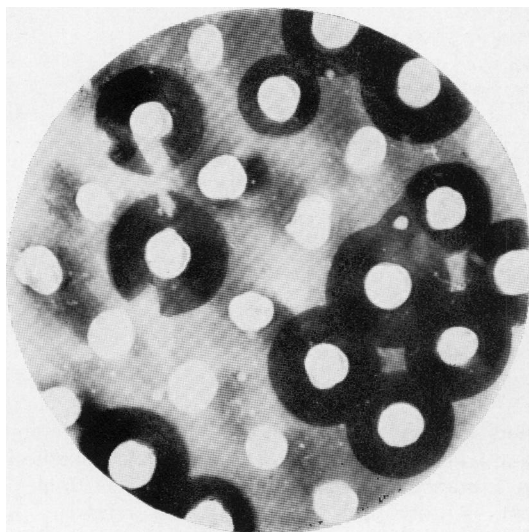

FIG. 1. *Lysozyme production by staphylococci on solid medium containing heat-killed* Micrococcus lysodeikticus *cells. Transparent zones around growing strains indicate lysis of substrate.*

Preparation of the substrate (*M. lysodeikticus* suspension) *M. lysodeikticus* strain Fleming was obtained from the Strain Collection, State Institute of Hygiene, and grown on Smolelis and Hartsell medium (Smolelis and Hartsell, 1949).

The bacterial suspension was autoclaved (15 min, 1 atm) and, after cooling, added to the liquefied medium to make a final concentration of about 10^9 cells per millilitre of the medium. The medium was poured on Petri plates 10 cm in diameter and, after solidification, the plates were dried for two hours at 37°C; they could be stored for several days in a refrigerator.

PROCEDURE The medium was inoculated with a drop of six-hour broth culture of each of the strains tested; 20 to 25 drops were put on one plate. Readings were made for the first time after 24 hours' incubation at 37°C, and a second time after 48 hours. Lysozyme-producing strains were surrounded by a transparent zone, which was best visible in a dark background. A test plate with the strains tested and with zones visible around some of them is illustrated in Figure 1.

DETERMINATION OF STAPHYLOCOCCAL LYSOZYME PRODUCTION BY TEST TUBE METHOD The procedure was based on the method reported by Kashiba, Niizu, Tanaka, Nosu, and Amano (1959) as described in another paper (Hawiger, 1968).

The strains tested were inoculated into test tubes with Difco brain-heart infusion medium and incubated for 24 hours at 37°C on a shaking machine. The activity of the lysozyme produced was determined in the supernatant by adding 2 ml to 2 ml of the substrate (*M. lysodeikticus* standard suspension) and calculating the percentage of the optical density (index of lysis) at 550 mμ wavelength. A reduction in the optical density of the *M. lysodeikticus* standard suspension higher than 10% was accepted as a positive result, indicating the effect of lysozyme.

RESULTS

A total of 543 strains was tested, including 503 strains of *S. aureus*, 35 strains of *S. epidermidis*, and five strains of cocci not belonging to the genus Staphylococcus (catalase + glucose fermentation in the OF test); the latter were eliminated from further analysis. They produced no lysozyme. Lysozyme was produced by all *S. aureus* strains, irrespective of their origin. Among 35 *S. epidermidis* strains, 13 produced lysozyme. Strains found to produce no lysozyme by the plate method were negative also by the test tube method.

In the set of strains tested, the production of several staphylococcal agents was compared, namely, free coagulase, clumping factor, staphylokinase, Tween 80-lipase, and lysozyme. $HgCl_2$ resistance, was also determined. The results are presented in Table I.

TABLE I

LYSOZYME PRODUCTION COMPARED WITH OTHER PROPERTIES OF THE STAPHYLOCOCCI TESTED

Oirgin of Strains	*Lysozyme-producing Strains*						*Non-lysozyme-producing Strains*						*Total*
	Total	*Free Coagulase*	*Bc*	*Staphylokinase*	*Tween 80 Lipase*	*$HgCl_2$ Resistance*	*Total*	*Free Coagulase*	*Bc*	*Staphylokinase*	*Tween 80 Lipase*	*$HgCl_2$ Resistance*	
Sanitary-epidemiological stations (routine diagnosis)	136	134	130	130	67	70	4	—	—	—	—	—	140
Clinic of Surgery (bun patients)	175	171	173	134	78	119	—	—	—	—	—	—	175
Collection at this laboratory	171	148	149	120	75	41	11	—	—	—	—	—	182
Routine diagnostic work of this laboratory	34	31	31	31	28	19	7	—	—	—	2	—	41
Total	516	484	483	410	248	249	22	—	—		2	—	538

TABLE II

INCIDENCE OF LYSOZYME PRODUCTION BY STAPHYLOCOCCI

Author	S. aureus		S. epidermidis		*Other Properties Tested*
	Lysozyme (+)	*Lysozyme* (−)	*Lysozyme* (+)	*Lysozyme* (−)	
Welsch (1959)	30	—	—	—	—
Kashiba *et al.* (1959)	55	—	11	31	Sensitivity to leukozyme A
Omori *et al.* (1960)	133	—	3	200	—
Goldbach and Haenel	82	—	15	28	Protease
Jay (1966)	120	6	4	45	Egg yolk factor Rabbit haemolysin Sheep haemolysin
Total	420	6	33	304	
Material tested in the present study	503	—	13	22	Staphylokinase, Tween-80 lipase, $HgCl_2$ resistance

Out of 516 lysozyme-producing strains, free coagulase was produced by 484 (93 %), clumping factor by 483 (93 %), staphylokinase by 410 (79 %), lipase by 248 (48 %) strains, and 249 (48 %) strains were resistant to $HgCl_2$. Among 22 strains producing no lysozyme, only two produced lipase.

DISCUSSION

A total of 426 coagulase-positive strains had been tested by all the authors who reported lysozyme production by staphylococci. The material tested in the present study included 503 coagulase-positive strains. A comparison of the results obtained with data reported by various other authors (Table II) suggests a significant coincidence as regards lysozyme production by *S. aureus*.

Almost beyond doubt, lysozyme is produced by all strains of this species. The question of lysozyme production by coagulase-negative strains remains controversial. According to the taxonomic criteria valid for *S. epidermidis*, especially in view of the existence of many other coagulase-negative cocci, strains were collected of which about one third were lysozyme-producing staphylococci. This quantity is in accordance with the percentage reported by Goldbach and Haenel (1964), who tested a similar total number of strains, and a little higher than the data reported by Kashiba *et al.* (1959). It should be mentioned that lysozyme production was determined under similar conditions in the present study and in that reported by Goldbach and Haenel, while Kashiba *et al.* determined it in broth culture by the test tube method.

The applied plate method of lysozyme determination appeared to be convenient and rapid for detecting this enzyme in staphylococci. Of the strains found to produce no lysozyme in the plate method, none produced it under the conditions of liquid culture in brain-heart infusion. As compared with the test tube method, the plate method has a number of advantages. It is easier to perform. Autolysin, which might be released in staphylococcal cultures, is not a source of difficulty, for its production is very low in solid medium (Ralston, Lieberman, Baer, and Krueger, 1957). *M. lysodeikticus* autolytic enzymes also do not interfere in the determination by the plate method, being inactivated by higher temperatures before testing.

Lysozyme production seems to be a property more frequent than $HgCl_2$ resistance and the production of free and bound coagulase, staphylokinase, and lipase. None of the agents, except coagulase, has been previously tested comparatively with lysozyme. Except for two lipase-producing strains, lysozyme-negative strains do not produce other agents tested.

It is not clear why lysozyme is produced by some *S. epidermidis* strains. According to Japanese authors (Kashiba *et al.*, 1959), most coagulase-negative and lysozyme-producing strains are resistant to leukozyme A, and thus have a chance of surviving inside leukocytes. On the other hand, Jay's results (Jay, 1966) suggest that coagulase-negative strains isolated from cases of subacute endocarditis, infections of the urinary tract, and wounds produce no lysozyme although they are considered pathogenic. Unfortunately, resistance of these strains to leukozyme A was not tested by Jay so that final conclusions are not possible.

Beyond doubt lysozyme production by staphylococci is more frequent than the production of coagulase and other agents. It can be determined under the conditions of routine bacteriological diagnosis; colonies producing no lysozyme are unlikely to be coagulase positive, but the reverse does not hold and all lysozyme producers must be tested further for evidence of potential pathogenicity.

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