

"transformation"
might have been a phase.
no quant. data.

1/25/47
2/22/47

as follows.

VARIATION IN PIGMENT PRODUCTION IN STAPHYLOCOCCUS AUREUS¹

PAMELA H. BYATT, GREGORY J. JANN, AND A. J. SALLE

Department of Bacteriology, University of California, Los Angeles, California

Received for publication February 14, 1948

The possibility of transforming a specific type of pneumococcus into another type has been recognized since 1928 when Griffith first described this phenomenon. He succeeded in transforming, *in vivo*, an attenuated and nonencapsulated variant into a virulent encapsulated strain of a heterologous specific type. Dawson and Sia (1931) were able to transform pneumococcus types *in vitro*. They used growing R cells in a medium containing anti-R serum and heat-killed encapsulated cells of another type. Later Alloway (1932, 1933) was able to obtain specific conversion of pneumococci by the use of filtered crude extracts of S cells. Berry and Dedrick (1936) converted the virus of rabbit fibroma into that of infectious myxoma in an experiment analogous to that used in pneumococcus transformation. Except for these two specific instances there appears to have been no mention in the literature of attempts at transforming other microorganisms using specific extracts.

During the past few years attempts have been made to isolate and identify the transforming substance. The results of this work indicate that the substance has the characteristics of a desoxyribonucleic acid (Avery, MacLeod, and McCarty, 1944; McCarty, 1946; McCarty and Avery, 1946).

The present study deals with an attempt to induce transformation of a white *Staphylococcus aureus* into a chromogenic type. There are no reports of this transformation having been induced experimentally by a chemically defined substance. However, Pinner and Voldich (1932) do report the reversion of *Staphylococcus albus* into *S. aureus* by growth of the organism in antialbus serum. Nutini and Lynch (1946) were able to induce a strain of *S. aureus* to lose its ability to produce pigment, and during two and one-half years of observation it did not revert to the original orange growth.

EXPERIMENTAL PROCEDURES

Staphylococcus aureus ATC 6538 was chosen as the source material for any possible transforming substance since it is a highly chromogenic organism. *S. aureus* Smith was used as the test organism for conversion; it was nonchromogenic and decidedly mucoid in colony form, and varied in biochemical reactions from the 6538 strain. The Smith strain has been observed over a period of 10 months

¹ After this work was completed an article appeared in the *Bull. acad. med.*, **131**, 39, 1947, by Boivin, Vendrely, and Tulasne, in which the authors stated that they were able to bring about a "directed mutation" of a coliform strain from one antigenic and fermentative type to another by growth in the presence of desoxyribonucleic acid derived from a strain possessing the new characters. Material from the smooth form only was effective.

P-276 ↓ T16

with no visible evidence of chromogenesis. Tests for chromogenic colonies were made on plates of yeast extract agar and on plates of Chapman-Stone medium (1946). Serial passage was made through 10 mice, but again there was no change in the color of colonies.

Development of chromogenicity by the colonies of a test organism was accepted as possible evidence of conversion. This conclusion was based on the fact that there seems to be no report in the literature of a white *Staphylococcus* spontaneously becoming chromogenic *in vitro*.

The test organisms were checked for colony type, ability to liquefy gelatin, to ferment certain sugars, and to produce coagulase. Reactions in litmus milk were also observed (table 1).

The requirements for transformation being unknown, certain cultural conditions suggested by Avery, MacLeod, and McCarty (1944) in their work with pneumococcus transformation were accepted. These conditions were found necessary for the conversion of pneumococcal types *in vitro*. The components included in the transformation test will be described in the following order: (1) infusion broth; (2) serum; (3) strain of test organism; and (4) method of preparing the staphylococcal extract.

TABLE 1
Cultural reactions of the test organisms

ORGANISM	PIGMENT	GELATIN LIQUEFACTION	COAGULASE	FERMENTATION		LITMUS MILK
				Lactose	Mannitol	
<i>S. aureus</i> Smith . . .	Grayish white	+	+	-	+	Peptonized
<i>S. aureus</i> 6538	Golden	+	+	+	+	Acid

Infusion broth. Difco beef heart infusion broth, adjusted to an initial pH of 7.2 to 7.4, was used as the basic medium. The broth was first adsorbed with charcoal according to the method described by MacLeod and Mirick (1942) before being autoclaved in 250-ml quantities. This procedure, originally initiated for the removal of sulfonamide inhibitors, has been reported by Avery, MacLeod, and McCarty (1944) as eliminating to a large extent unpredictable variations in individual lots of broth.

Serum. Beef serum, filtered through a Mandler filter, was used routinely in conjunction with the infusion broth. The proportions of serum to broth were 1 to 3, and this mixture was used for maintaining the stock cultures as well as for testing for transforming activity. In order to destroy any enzyme that might interfere with a transforming principle the serum was heated to 60 C for 30 minutes.

Test organisms. The organisms used in testing for transformation were grown in two series, one in serum broth and the other in plain adsorbed infusion broth; serial transfers were made daily. The Smith strain of *Staphylococcus aureus* showed colonies that were round, raised, opaque, glistening, and so mucoid that colonies touched with an inoculating needle would lift off the agar surface in one

mass or pull out in long mucoid threads. On yeast extract agar these colonies were an off white in color. Sometimes a few dissociant colonies were found on a plate. These, however, were not so opaque nor did they have the characteristic mucoid quality.

S. aureus 6538, the organism from which extracts were made, showed an intense orange chromogenesis on Chapman-Stone medium; the color was not quite so intense on yeast extract agar. The colonies were round, raised, and shiny, with an entire margin.

Method of preparing extract. A 48-hour chromogenic slant of *S. aureus* 6538 was washed off with broth and this broth suspension used to inoculate Blake bottles. Yeast extract agar was used as the base medium. The Blake bottles were incubated for periods varying from 18 to 24 hours and accepted for further treatment if there was visible evidence of chromogenesis on the slant. Growth on each slant was then washed off in 10 ml of a solution containing 0.1 M sodium chloride and 0.1 M sodium citrate. McCarty and Avery (1946) used this solution to inactivate desoxyribonuclease that had been released with the breakdown of the pneumococcus cell.

Some method had to be devised for rupturing the staphylococcal cells without inactivating any transforming principle that might be present. Chemical agents were tried and seemed to be of slight, if any, value in lysing heavy suspensions of staphylococci. The mechanical method of rapid freezing and thawing was used.

The citrate-saline suspension of organisms was dispensed in sterile test tubes; approximately 6 ml were in each tube. The freezing bath consisted of a beaker, the outside of which was insulated with nonabsorbent cotton. Sufficient amyl alcohol, to more than cover the tube containing the bacterial suspension, was added to the beaker, and pieces of solid CO₂ were placed in the alcohol to hold the temperature well below freezing. The tubes of bacterial suspension were immersed in the freezing bath until frozen solid, removed, and placed in warm water to thaw, then frozen again. This procedure was repeated over a period of 2 hours. At the end of this time gram stains still revealed an abundance of intact gram-positive organisms. Subculturing also indicated that many viable organisms remained. The treated saline-citrate suspension was then deproteinized by a slight modification of the method of Sevag, Lackman, and Smollens (1938). Approximately 5 ml of a chloroform-amyl-alcohol mixture (4 ml chloroform to 1 ml amyl alcohol) were added to each 10 ml of bacterial suspension. The whole was then shaken in a Kahn shaker for 30 minutes, centrifugated for 20 minutes, and the chloroform emulsion removed. The supernatant was again extracted with chloroform-amyl-alcohol, and both fractions were pooled in a separatory funnel. The addition of 2 volumes of alcohol to the extract resulted in flocculation of a fair amount of white material. Citrated saline was then added to the alcohol extract and the whole was well shaken. The funnel was placed in the refrigerator and left there overnight to permit separation of the two phases. The saline extract, which was decanted the following morning, was faintly cloudy, with cloudiness increasing slightly in density as the solution attained room temperature. It is this extract that was used in testing for transforming activity.

Qualitative chemical tests. The saline extracts gave negative biuret, Millon, and xanthoproteic tests. Benedict's test for reducing sugars was also negative. The orcinol test (Bial) for pentoses was faintly positive.

Determination of biological activity. In an attempt to obtain optimum proportions, varying quantities of saline extract were added to a series of tubes each containing the same amount of serum broth. Each tube in a series was inoculated with a loopful of an 18- to 24-hour broth culture of the test organism. The tubes were incubated at 37 C for 24 hours. At the end of 24 hours a streak plate, on yeast extract agar, was made from each dilution. A loopful of each dilution culture was transferred to another tube containing the same quantity of extract. With each determination this procedure was repeated for 5 days in series. Control plates of the test organism grown in serum broth were made each day. The

TABLE 2
Serial transfer of test organism in dilutions of extract

TUBE NO.	EXTRACT	APPROXIMATE PERCENTAGE OF CHROMOGENIC COLONIES PER PLATE				
		1st day	2nd day	3rd day	4th day	5th day
	<i>ml</i>					
1	0.05	—	—	—	—	—
2	0.15	—	—	—	1	3
3	0.25	5	3	1	—	3
4	0.35	—	—	—	30	40
1	0.15	—	—	70	—	—
2	0.20	—	—	—	—	—
3	0.25	—	—	20	50	—
4	0.30	—	—	40	—	—
1	0.15	—	—	—	—	—
2	0.20	—	—	—	—	—
3	0.25	—	—	—	—	—
4	0.30	—	—	—	—	—
1	0.17	—	—	—	—	—
2	0.25	—	—	—	—	—
3	0.32	—	—	50	70	60
4	0.40	—	—	—	—	—

— = no pigmented colonies seen on the plate.

plates were grown at 37 C for 48 hours, examined for chromogenic colonies, and then kept at room temperature for 2 weeks before being discarded as negative. A stock slant of yeast extract agar was made of any suspected colony, and further studies were made with this stock strain.

RESULTS

Pigmented colonies were obtained by growing the white *Staphylococcus aureus* Smith in extracts prepared from the highly chromogenic 6538 strain of *S. aureus*. Transformation was sporadic and did not occur in all cases. In some series no chromogenic colonies developed; in others pigmented colonies were found on plates made from inoculated extract dilutions of the first to the fifth day. The results in table 2 are from experiments selected for illustration.

Whether the stock culture, used to inoculate extract dilutions, was grown in serum broth or in plain adsorbed broth seemed to have little effect on the results. In many cases colonies did not show pigmentation until there had been a period of incubation at room temperature. A few of the chromogenic colonies were round and smooth, differing from the control colonies only in color. The majority of colonies showed an undulating margin that was more highly pigmented than the body of the colony. Frequently small chromogenic outbursts seemed to develop from a cream parent colony, and transfers made from this margin grew as round pigmented colonies. In no instance was chromogenesis so intense in the new strains as it was in control plates of *S. aureus* 6538; this fact was particularly evident on plates of Chapman-Stone medium. The new ability to produce pig-

TABLE 3
Cultural reactions of the isolated strains

STRAIN	AMOUNT OF PIGMENT	GELATIN LIQUEFACTION	COAGULASE	FERMENTATION			LITMUS MILK
				Glucose	Lactose	Mannitol	
Smith	—	+	+	+	—	+	Peptonized
6538	++++	+	+	+	+	+	Acid
534	++	+	+	+	—	+	Peptonized
755	++	+	+	+	—	+	"
1123	+	—	—	+	—	+	No reaction
1334	+	—	—	+	—	+	" "
1345	+	+	+	+	—	+	Peptonized
1355	++	+	+	+	—	+	"
1353	+	+	+	+	—	+	"
1822	+	+	+	+	—	+	"
1831	++	+	+	+	—	+	"
1833	+	+	+	+	—	+	"
1834	++	+	+	+	—	+	"
1843	++	+	+	+	—	+	"
1853	++	+	+	+	—	+	"
1854	++	+	+	+	—	+	"

ment proved, on ordinary serial transfer, to be unstable, many of the daughter colonies reverting to the original Smith type.

In studies of the newly isolated pigmented strains the colony type resembled those of the Smith organism. There was no variation in sugar reactions. Fermentation of glucose and mannitol was positive, and of lactose negative. Gelatin was liquefied and coagulase was produced by all but two of the least pigmented strains (table 3).

CONCLUSIONS

It has been found possible to stimulate or initiate chromogenesis in a nonchromogenic *Staphylococcus* by growing it in extracts of a pigmented strain of the same species. This fact suggests that there might be a transforming principle in the extracts analogous, but not identical, to the transforming principle used to convert *R. pneumococci* into specific types. The extracts used in either case seem

to have similar chemical properties. Both give negative protein tests and weakly positive tests for pentose (Bial).

A very small quantity of the transforming substance is capable of initiating a reaction. There is no explanation for the fact that some cells of the test organism are refractory to stimulation whereas others are not.

SUMMARY

An extract has been prepared from *Staphylococcus aureus* 6538, a highly chromogenic organism, which has the potentiality of inducing or stimulating chromogenesis in colonies of a white strain of the same species.

The extract prepared gives negative Millon, xanthoproteic, and Benedict tests, and a slightly positive test for pentose (Bial).

The newly isolated chromogenic organisms retain the biochemical properties of the parent culture, differing only in pigment production.

The intensity of pigment production is not so great in the new strains as in the strain from which the extract was prepared nor does it seem to be lasting.

REFERENCES

- ALLOWAY, J. L. 1932 The transformation *in vitro* of R pneumococci into S forms of different specific types by the use of filtered pneumococcus extracts. *J. Exptl. Med.*, **55**, 91-99.
- ALLOWAY, J. L. 1933 Further observations on the use of pneumococcus extracts in effecting transformation *in vitro*. *J. Exptl. Med.*, **57**, 265-278.
- AVERY, O. T., MACLEOD, C. M., AND McCARTY, M. 1944 Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exptl. Med.*, **79**, 137-158.
- BERRY, G. P., AND DEDRICK, H. M. 1936 A method for changing the virus of rabbit fibroma (Shope) into that of infectious myxomatosis (Sanarelli). *J. Bact.*, **31**, 50-51.
- CHAPMAN, G. H. 1946 The staphylococci. *Trans. N. Y. Acad. Sci.*, **9**, 52-55.
- DAWSON, M. H., AND SIA, R. H. P. 1931 *In vitro* transformation of pneumococcal types. I. A technique for inducing transformation of pneumococcus types *in vitro*. *J. Exptl. Med.*, **54**, 681-699.
- GRIFFITH, F. 1928 The significance of pneumococcal types. *J. Hyg.*, **27**, 112-159.
- McCARTY, MACLYN 1946 Chemical nature and biological specificity of the substance inducing transformation of pneumococcal types. *Bact. Revs.*, **10**, 63-71.
- McCARTY, MACLYN, AND AVERY, O. T. 1946 Studies on the chemical nature of the substance inducing transformation of pneumococcal types. II. Effect of desoxyribonuclease on the biological activity of the transforming substance. III. An improved method for the isolation of the transforming substance and its application to the pneumococcus types II, III, IV. *J. Exptl. Med.*, **83**, 89-104.
- MACLEOD, C. M., AND MIRICK, G. S. 1942 Quantitative determination of the bacteriostatic effect of the sulfonamide drugs on pneumococci. *J. Bact.*, **44**, 277-287.
- NUTINI, L. G., AND LYNCH, E. M. 1946 Further studies on the effects of tissue extracts on *Staphylococcus aureus*. *J. Exptl. Med.*, **84**, 247-261.
- PINNER, M., AND VOLDICH, M. 1932 Derivation of *Staphylococcus albus*, *citreus* and *roseus* from *Staphylococcus aureus*. *J. Infectious Diseases*, **50**, 185-202.
- SEVAG, M. G., LACKMAN, D. B., AND SMOLLENS, J. 1938 The isolation of the components of streptococcal nucleoproteins in serologically active form. *J. Biol. Chem.*, **124**, 425-436.