Mutants with Impaired Respiration in *Staphylococcus afermentans*

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SUMMARY

Mutants with small colonies and impaired respiration were induced by 5-fluorouracil in cultures of *Staphylococcus afermentans*, NCTC 7503. The consumption of oxygen in colourless mutants attained 66–77 %, and in mutants with orange colonies 68–77 % of normal values. Inhibitors of protein synthesis (chloramphenicol, tetracycline, chlortetracycline), inhibitors of cell-wall synthesis (penicillin G, phenoxymethylpenicillin), as well as an inhibitor of RNA formation (actinomycin C) affected the growth of the parent and mutant staphylococci to the same extent. On the other hand, inhibitors which affect DNA—namely, degranol, myleran-mannitol and mitomycin C—selectively inhibited the growth of mutant staphylococci with impaired respiration. Mitomycin C induced irreversible depolymerization of DNA in bacterial cells; colourless mutants were 2·7–3·5 times, and mutants with orange colonies 33–45 times more vulnerable to its action than the parent culture. The extraction of DNA from bacterial cells showed that the capacity of DNA for extraction from a complex with proteins was decreased in colourless mutants by 2·8–2·9 times, and in mutants with orange colonies by 5·0–7·3 times.

INTRODUCTION

Mutants with impaired respiration in *Staphylococcus aureus*, induced by ultra-violet radiation (Gause, Kochetkova & Vladimirova, 1957), have been studied in some detail (Gause, Kochetkova & Vladimirova, 1961). These mutants were selectively inhibited by substances which affected the nucleic acids (Gause & Kochetkova, 1962). In this connexion the induction of mutants with 'small colonies' and impaired respiration in some other species of micrococci, and a comparative investigation of their characters, are highly desirable. Some new results along these lines obtained in the work with *Staphylococcus afermentans* are described here.

METHODS

Organisms. The original culture of *Staphylococcus afermentans* was obtained from the National Collection of Type Cultures—NCTC 7503. It was isolated during the course of a routine examination of an eye swab from an 11-year-old boy in the Public Health Laboratory at Wakefield, Yorkshire, in 1948.

Measurement of bacteriostatic action of chemical agents. The methods used, as well as composition of the nutrient media, were described earlier in this Journal (Gause & Kochetkova, 1962).

Manometry. Manometric experiments were carried out in a conventional Warburg apparatus under aerobic conditions at 37°. The organisms were grown on

nutrient agar in Petri dishes for 24 hr. at 37°, washed twice on a centrifuge with 0.8 % NaCl, and afterwards suspended in 0.8 % NaCl in the concentration of about 5 % w/v (wet weight). The dry weight of bacterial suspensions was also estimated, and used in the calculation of oxygen consumption. Suspensions of bacteria immediately before experiments were diluted ten times with M/15 phosphate buffer ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} + \text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.4) containing 0.5 % of glucose.

Estimation of nucleic acids. Bacteria contained in 100 ml. of medium were washed by centrifugation, and ribonucleic acid (RNA) was extracted from sedimented cells by 1.5 ml. 10 % perchloric acid in the cold (−4°) for 17 hr. The cells were again washed on the centrifuge with 0.5 ml. fresh cold 10 % perchloric acid. The extracts were combined and diluted by an equal volume of 10 % perchloric acid. These extracts did not contain deoxyribonucleic acid (DNA). RNA contents of extracts were estimated by the orcinol test as follows: to 1 ml. of the diluted extract were added 2.5 ml. 0.02 % FeCl_3 (in concentrated HCl) and 0.15 ml. 10 % orcinol (in 95 % EtOH). After the tube contents were mixed, the tube was heated for 15 min. in a boiling water bath, cooled, and read photometrically at 660 m μ .

After removal of RNA the bacterial cells were extracted with a fresh 1.5 ml. portion of 10 % perchloric acid at 90° for 15 min., and the contents of DNA in the extract were assayed by the diphenylamine method as follows: to 1 ml. of extract in a small tube were added 2.5 ml. of the diphenylamine reagent (1.0 g. diphenylamine dissolved in 2.0 ml. conc. H_2SO_4 and 98 ml. glacial acetic acid), and the tube was placed in a boiling water bath for 10 min. It was cooled and read photometrically at 540 m μ .

Extraction of DNA by Schmidt & Thannhauser procedure. The method used was that described by Spirin, Belozersky, Shugaeva & Vanushin (1957) as a modification of the original procedure (Schmidt & Thannhauser, 1945). In our experiments 500 mg. of dry bacteria (previously washed by alcohol and ether until washings were colourless) were suspended in 4 ml. 0.5N-NaOH, and hydrolysed at 37° for 18 hr. The suspension was then acidified in the cold by 70 % HClO_4 to make the total concentration of perchloric acid in the solution equal to 1–2 %, and then centrifuged in the cold. The solution containing RNA was discarded, and the sediment containing DNA was washed twice with cold 0.2N- HClO_4 , once with alcohol and once with alcohol and ether (1+1) in the cold, and finally again with the cold 0.2N- HClO_4 to remove alcohol and ether. A volume of 10 ml. was used for washing at each stage. The wet sediment was neutralized with a saturated solution of NaHCO_3 , and then extracted by alkaline (pH 8) 10 % NaCl on the boiling water bath for 1 hr. The concentration of DNA in the extract was estimated spectrophotometrically by the difference of optical densities at 595 and 650 m μ . Spectrophotometer SF-4 (U.S.S.R.) was used in our work.

RESULTS

Induction of mutants with impaired respiration. Various mutagenic factors were tried, but positive results were observed only in experiments with 5-fluorouracil. The parent culture of *Staphylococcus afermentans* 7508 was grown at 37° in test tubes in nutrient broth containing 60–70 μg . 5-fluorouracil/ml., and samples from the tubes were daily streaked in various dilutions on plates of nutrient agar. After 4–5 days of incubation of the culture in broth, the subcultures on plates showed

the appearance of small colonies of mutants, dispersed among the colonies of normal bacteria. Some of the mutants were growing in the form of sectors in the colonies of normal organisms. Numerous mutants with small colonies were isolated in the course of this work, and classified into two groups: colourless colonies and colonies of orange colour in various shades. Of the former group, mutants 19 and 44 and of the second group mutants 42 and 22 were used in this study.

Table 1 shows that the consumption of oxygen in colourless mutants attained 66–77 %, and in mutants with orange colonies 68–77 % of normal values, i.e. respiration was impaired to the same degree in these mutants.

Table 1. *Oxygen consumption by washed cell suspensions of parent and mutant cultures of Staphylococcus afermentans in the presence of glucose*

	No. of experiments	Q _{O₂} *
Parent culture	12	82.5
Mutant 42 (orange colonies)	7	63.3
Mutant 22 (orange colonies)	5	56.2
Mutant 19 (colourless colonies)	5	54.5
Mutant 44 (colourless colonies)	5	63.9

* The consumption of oxygen in mm³/hr. calculated for 1 mg. dry weight of bacteria.

Effects of inhibitors. Measurement of minimum inhibitory concentrations of various compounds in nutrient broth showed that inhibitors of protein synthesis in the bacterial cell affected the growth of the parent and mutant organisms to the same degree (0.4–0.5 µg. chloramphenicol/ml.; 0.2–0.3 µg. tetracycline/ml.; 0.4–0.5 µg. chlortetracycline/ml.). Mutant and parent cultures were affected also to the same degree by inhibitors of cell-wall synthesis (0.03–0.05 unit penicillin G/ml.; 0.02–0.03 unit phenoxymethylpenicillin/ml.), as well as by 5-fluorouracil. Addition of thymine (100 µg./ml.) or uracil (100 µg./ml.) produced no effect upon the inhibitory action of fluorouracil.

We were particularly interested in the analysis of the inhibitory action of actinomycin C and mitomycin C, as far as these compounds selectively affect the formation of nucleic acids in the bacterial cell. Experiments showed that mitomycin C selectively inhibited formation of DNA, and actinomycin C selectively affected synthesis of RNA in the logarithmic phase of growth of *Staphylococcus afermentans* (Table 2).

It is of interest that actinomycin C inhibited the growth of parent and mutant cultures of *Staphylococcus afermentans* to the same degree; the minimal inhibitory concentration in broth attained 0.003–0.004 µg./ml. The estimations were made by the tube dilution method, as described by Gause & Kochetkova (1962). In distinction from this, the substances selectively affecting DNA formation—namely, mitomycin C, degranol (1,6-bis-(β-chloroethylamino)-1,6-deoxy-*D*-mannitol), and myleran mannitol (1,6-dimethanesulphonyl-*D*-mannitol) also selectively inhibited the growth of mutants with the impaired respiration (Table 3).

The action of mitomycin C is of particular interest, since this compound selectively and irreversibly depolymerizes DNA in bacterial cells (Reich, Shatkin & Tatum, 1961). It is therefore of significance that colourless mutants were 2.7–3.5 times, and mutants with orange colonies 33–45 times more vulnerable to the action

of this compound than the parent culture. It may be suggested that some alteration in the DNA of mutants with impaired respiration made the cells more vulnerable to the action of mitomycin C, and that these alterations are more fundamental in mutants with orange colonies as compared to colourless mutants.

Table 2. *Effect of Mitomycin C and Actinomycin C on the growth and nucleic acid synthesis of Staphylococcus afermentans**

Compound, $\mu\text{g./ml.}$	Incubation time, min.	Turbidity, E_{660}	RNA	DNA
			(orcinol reaction) E_{660}	(diphenylamine reaction) E_{540}
Control	0	0.190	0.411	0.091
	75	0.265	0.481	0.113
	150	0.337	0.578	0.132
Mitomycin C, 0.1	0	0.190	0.411	0.091
	75	0.270	0.487	0.086
	150	0.342	0.577	0.088
Control	0	0.171	0.399	0.085
	90	0.261	0.477	0.109
	180	0.369	0.581	0.136
Actinomycin C, 0.1	0	0.171	0.399	0.085
	90	0.209	0.359	0.092
	180	0.259	0.372	0.109

* The content of RNA and DNA in the microbial cells are given per 100 ml. of the culture. Turbidity E_{660} is proportional to the dry weight of bacteria. The figures give the average data of 3 series of experiments. Growth at 37° in Erlenmeyer's flasks. To 180 ml. of nutrient broth were added 20 ml. of 18 hr. culture of bacteria, and kept at 37° for 90 min. At this time compounds were added and the first sample taken, marked as '0 time'. For each analysis two samples of 50 ml. each were taken from two duplicate flasks, and mixed to make 100 ml.

Table 3. *Minimal growth inhibitory concentrations ($\mu\text{g./ml.}$) of various compounds in nutrient broth for Staphylococcus afermentans and its mutants**

Compound	Parent	Mutant 42 (orange)	Mutant 22 (orange)	Mutant 19 (colourless)	Mutant 44 (colourless)
Mitomycin C	0.0500	0.0015	0.0011	0.0140	0.0180
Degranol	210	22	28	25	29
Myleran-mannitol	4000	620	520	620	410

* The conditions of assays were described by Gause & Kochetkova (1962).

Differences in the DNA-protein relationship of normal and mutant organisms. The extraction of DNA from bacterial cells by Schmidt and Thannhauser procedure (in the modification described by Spirin *et al.* 1957; see Methods) showed that the average activity of extracts (D_{595} – D_{650}) in the parent culture attained 0.262, in mutant 19 (colourless) 0.094, in mutant 44 (colourless) 0.090, in mutant 42 (orange) 0.036, and in mutant 22 (orange) 0.052. In other words the capacity of DNA for extraction from a complex with proteins was decreased in colourless mutants by 2.8–2.9 times, and in mutants with orange colonies by 5.0–7.3 times.

Our observations refer to mild separation of DNA from proteins by extraction at pH 8 by 10 % NaCl, as prescribed by Schmidt & Thannhauser (1945). In distinction from this, an acid hydrolysis by 0.5N-HClO₄ at 70° for 20 min. (Ogur & Rosen, 1950)

completely separates DNA from proteins in normal and mutant cells of *Staphylococcus afermentans*, and differences between these cultures are no longer observed. It is difficult at present to interpret these observations, but it is interesting that Kirby (1961) found recently some differences in the DNA-protein complexes of normal and cancer cells.

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REFERENCES

- GAUSE, G. F., KOCHETKOVA, G. V. & VLADIMIROVA, G. B. (1957). Biochemical mutants of staphylococci with impaired respiration. *Dokl. Acad. Sci. U.S.S.R.*, **117**, 720.
- GAUSE, G. F., KOCHETKOVA, G. V. & VLADIMIROVA, G. B. (1961). Biochemical changes accompanying impaired respiration in staphylococci. *Nature, Lond.* **190**, 978.
- GAUSE, G. F. & KOCHETKOVA, G. V. (1962). Vulnerability of nucleic acids in mutant staphylococci. *J. gen. Microbiol.* **29**, 317.
- KIRBY, K. S. (1961). Nucleic acids and cancer. *Progr. exp. Tumor Res.* **2**, 291.
- OGUR, M. & ROSEN, G. (1950). The nucleic acids of plant tissues. I. The extraction and estimation of deoxypentose nucleic acid and pentose nucleic acid. *Arch. Biochem.* **25**, 262.
- REICH, E., SHATKIN, A. J. & TATUM, E. L. (1961). Bactericidal action of mitomycin C. *Biochim. biophys. Acta*, **53**, 132.
- SCHMIDT, G. & THANNHAUSER, S. J. (1945). A method for the determination of deoxyribonucleic acid, and phosphoproteins in animal tissues. *J. biol. Chem.* **161**, 83.
- SPIRIN, A. S., BELOZERSKY, A. N., SHUGAEVA, N. V. & VANUSHIN, B. F. (1957). A study of species specificity of nucleic acids in bacteria. *Biokhimiya (Moscow)*, **22**, 744.