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NUTRITIONAL MUTATIONS AND TRANSDUCTION BY ULTRAVIOLET-INACTIVATED PHAGE IN *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

CARERE, A., AND ISABELLA SPADA-SERMONTI (Istituto Superiore di Sanità, Rome, Italy). Nutritional mutations and transduction by ultraviolet-inactivated phage in *Staphylococcus aureus*. J. Bacteriol. **88**:226-232. 1964.—By use of a semisynthetic medium, various nutritional mutants of *Staphylococcus aureus* 80 were isolated. Most of them require purine derivatives. From their mutation pattern, it may be inferred that guanine can be converted to both guanilic and adenilic acid, and adenine to the latter only. Guanosine, hypoxanthine, and inosine appear to be intermediate in the synthesis of adenilic acid from exogenous guanine. Transduction of several nutritional markers was obtained by streaking phage 80 obtained from the donor strain onto selective media heavily seeded with the recipient strain. To prevent lysis, the phage was previously ultraviolet-inactivated.

Transduction in *Staphylococcus aureus* was first reported by Cavallo and Terranova (1955). The system has since been exploited by several authors, but has suffered for a long time from the lack of a proper minimal agar medium, which has prevented the use of nutritional markers. Edgar and Stocker (1961) recently obtained the transduction of synthetic abilities between wild-type isolates with different nutritional requirements. Ritz and Baldwin (1962) obtained tryptophan-dependent mutants on semisynthetic medium based on acid-hydrolyzed casein, which lacks tryptophan. The same medium has been used to select transductants.

The present report deals with the isolation of several types of nutritional mutants from *S. aureus* strain 80 by the use of a similar semisynthetic medium and the transduction of the wild alleles by means of the virulent typing phage 80. To prevent lysis of the transductants, the phage suspension was previously ultraviolet-inactivated.

MATERIALS AND METHODS

Strains. Throughout the work *S. aureus* 80 (Williams), as well as its virulent typing phage 80 of serological group B (Rountree, 1949), were used. Both were kindly supplied by Vittorio Ortali of the Microbiology Laboratories of our Institute.

Media. The bacterial strains were kept on slants of nutrient agar (Difco). Trypticase Soy (TS) Broth (BBL) was used for bacterial growth; 0.004 M $CaCl_2$ was added for propagation of phage stocks and phage assays. TS agar medium was prepared by adding 1.5% (w/v) agar to TS Broth; TS soft agar medium was prepared by adding 0.5% (w/v) agar. Nutrient broth (Difco) containing 0.25% (w/v) K_2HPO_4 and 0.20% (w/v) glucose (BPD) was used for preparation, dilution, and storing of phage stocks. For some transducing experiments Brain Heart Infusion (BHI) Agar containing 0.5% (w/v) sodium citrate was employed. BHI soft agar was prepared by adding 1% (w/v) agar. For isolation and transduction of nutritional markers, a modified version of the minimal medium (M) of Ritz and Baldwin (1962), lacking purine and pyrimidine bases which are not required for growth of *S. aureus* 80, was used.

Supplements to M medium were added, when required, at the following concentrations per liter: purine bases and their nucleosides, 20 mg (except adenine, 50 mg); uracil, 100 mg; choline, 1 mg; riboflavine, 20 mg. A complete medium (C) for auxotroph isolation was prepared by adding to the M agar 20 ml of hydrolyzed yeast nucleic acid and 10 ml of a vitamin solution (according to Pontecorvo et al., 1953).

Isolation and characterization of mutants. Samples of 0.1 ml of a bacterial suspension containing ca. 10^4 cells were spread on plates of C medium and treated to 300 ergs per mm^2 with a Sylvania high-pressure mercury sterilizing lamp. After 24 hr of incubation at 37 C, the surviving colonies

were gently replicated with a velvet pad (Lederberg and Lederberg, 1951) onto dishes of fresh M medium, and the auxotrophic mutants unable to give rise to growth on M medium were scored as colonies on C medium. The mutant clones were characterized by the auxanographic technique or by serial streaks on semisynthetic media supplemented by purines, pyrimidines, or vitamins, either in pools or singly. Drug-resistant mutants were selected among untreated cells plated at high densities onto drug-supplemented media.

Dilutions. In addition to the standard method, a simple device developed by one of our technicians (G. Conti) was employed. This device makes use of the "fish spine" beads currently used in biological assays. By touching the surface of the liquid to be diluted with the edge of a sterile bead held in pincers, 0.02 ml are sampled. The filled bead is dropped into 2 ml water and a 1:100 dilution is obtained. Conti's method is particularly suitable for virulent microorganisms.

Propagation of phage stocks. The agar layer method (Swanstrom and Adams, 1951) was routinely used. TS agar was used as a basal layer, and TS soft agar, to which 0.75 ml of bacterial culture (ca. 5×10^9 cells per ml) and 0.1 ml of phage suspension were added, was dispensed on top in 3-ml amounts. After ca. 15 hr of incubation at 30 C, 4 ml of BPD broth were poured on top, the soft agar was scraped with a loop, and the dish was further incubated at 37 C for 45 min. The phage crop was used for harvesting a second crop, and usually a third cycle was required to obtain a final titer of about 10^{13} phage per ml. Finally, the phage suspension was centrifuged at 7,000 $\times$ *g* for 10 min, Seitz-filtered, and stored at 4 C.

Inactivation of phage. The phage suspensions in BPD broth were treated by ultraviolet rays (see above) in a thin layer in the bottom of a small petri dish, gently agitated by hand at 10 cm from the ultraviolet tube. The dose (ergs per mm^2) was calculated with the ultraviolet dosimeter of Latarjet (1953) at the surface of the liquid. Because of absorption of ultraviolet light by the BPD broth, the dose at the cell level is somewhat overestimated.

Transduction by use of streptomycin-resistance markers. The procedure employed by Ritz and Baldwin (1961) for transduction of capacity to produce penicillinase was adopted. The transduction mixture, after centrifugation, was resuspended in BHI broth; 0.1 to 0.2 ml of the mixture was added to 2.5 ml of melted BHI soft agar and poured on a layer of BHI agar. After 2 or 4 hr, a third layer of 2.5 ml of BHI soft agar containing 330 μg of streptomycin was poured on top. Transductants were scored after 2 days of incubation at 37 C.

Transduction by use of nutritional markers. The recipient strain was grown for 24 hr on the surface of a slant of nutrient agar. The whole culture was then transferred into 2.5 ml of TS broth and shaken at 37 C for 2 to 3 hr in a large tube or in a small Erlenmeyer flask. The culture was then centrifuged at 7,000 $\times$ *g* for 15 min and resuspended in 1 ml of liquid M medium; 0.15 ml of the suspension (containing ca. 10^{12} cells per ml) was then spread on dishes of M medium (10 cm, internal diameter). After drying, circles (3 cm, diameter) were printed in the agar with the cutting edge of an empty sterile cylinder. A loopful of phage, from an ultraviolet-treated suspension (containing ca. 10^{13} particles per ml) was then spread on each of the small discs cut in the agar. Transductants were scored after overnight growth (Fig. 1). For the quantitative evaluation of the multiplicity of infection and of the rate of transduction, the volumes of the bacterial suspension and phage suspension spread on each disc were calculated as follows. Each disc represented one-tenth of the total surface of the petri dish,

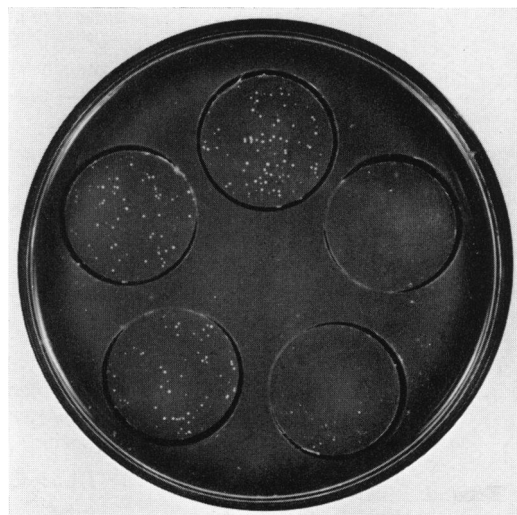

FIG. 1. *Spot test of transduction of the ura$^+$ marker by phage 80, on M medium. From 2 o'clock, clockwise, phage treated with 0, 4,000, 8,000, 16,000, and 32,000 ergs/mm^2 of ultraviolet light.*

thereby containing 0.015 ml of bacterial suspension. A loopful of phage, in a calibrated loop, contained 0.01 ml. Although not very accurate, this procedure gave satisfactory results at this stage of analysis.

RESULTS

Isolation of nutritional mutants. A total of 38 nutritional mutants were isolated and characterized. Three of them required vitamins (two riboflavine and one choline), two required uracil, two required either uracil or guanine, and all the others required purine derivatives. The mutations obtained are listed in Table 1. No amino acid-requiring mutants were isolated, because of the presence of hydrolyzed casein and cysteine in the minimal medium. Tryptophan was not present in the C medium.

TABLE 1. *Auxotrophic mutants obtained in Staphylococcus aureus 80*

Code of mutation	No. of mutants obtained	Supplement required
rib	2	Riboflavine
cho	1	Choline
ura	2	Uracil
pur/ura	2	Guanine *or* hypoxanthine *or* inosine *or* guanosine *or* uracil
purA	17	Guanine *or* hypoxanthine *or* inosine *or* guanosine
purB	1	Guanine *or* hypoxanthine *or* inosine *or* guanosine *or* xanthine
purC	1*	Guanosine *or* hypoxanthine *or* inosine *or* (guanine + adenine) *or* (guanine + adenosine)
Double mutants from *purA* strains‡		
purA purD	7	Guanosine *or* hypoxanthine *or* inosine *or* (guanine + adenine) *or* (guanine + adenosine)
purA purE	3	Hypoxanthine *or* inosine *or* (guanine + adenine) *or* (guanine + adenosine)
purA purF	1	Inosine *or* guanosine *or* (guanine + adenine) *or* (guanine + adenosine)
Total	38	

* Derived from a *ura* strain. Uracil still required.

‡ Selected for inability to grow on M medium + guanine.

The purine-requiring mutants allow some speculation on the pathway of purine nucleotide biosynthesis from preformed purines in *Staphylococcus*. Most of the primary mutants (18 of 19) grew on M medium supplemented by guanine, and none grew on M medium plus adenine. All these mutants may be assumed to be blocked in the de novo purine biosynthesis, and guanine but not adenine appears to be the preformed purine derivative utilized for the synthesis of purine nucleotides in the absence of the normal synthesis.

All the primary guanine-requiring mutants also grew well in the presence of hypoxanthine, guanosine, or inosine, but all (with the exception of one) grew very poorly with xanthine (or xanthosine) as the only supplement. Double mutants obtained from guanine-requiring auxotrophs, with a reduced ability in the utilization of preformed purines, all grew in media containing both adenine (or adenosine) and guanine, thus appearing to be blocked in some step of the interconversion of guanine into adenine derivatives. The least exacting (seven of ten) were able to utilize guanosine (or hypoxanthine or inosine) as the sole supplement in place of both adenine and guanine, and three more exacting ones utilized hypoxanthine or inosine, but not guanosine.

Transduction of nutritional markers with ultraviolet-inactivated phage. To select for transductants on M media, on which extensive lysis occurred, we attempted ultraviolet-inactivation of plaque-forming ability of phage 80, before carrying out the transduction experiments. This had been successfully used with a virulent mutant of temperate phage PLT-22 in *Salmonella typhimurium* by Goldschmidt and Landman (1962).

Phage 80, obtained from the strain designed to act as donor, was treated with ultraviolet light at various doses from 1,000 to 32,000 ergs per mm^2. Loopsful of the phage suspension with different survivals were immediately spread onto discs cut on the surface of an agar plate heavily seeded with the strain designed to act as recipient. M agar supplemented with the substance(s) required by the recipient was used to test the virulence of the

treated phages, and unsupplemented M agar was used to test the transducing ability of the phages. On the supplemented M agar, growth occurred, after overnight incubation, on the whole plate, but complete lysis was observed corresponding to spots of untreated phage or of phage treated with 4×10^3 ergs per mm² of ultraviolet light (phage survival, 10^{-1} to 10^{-2}). Incomplete lysis occurred in correspondence with phage treated with 8×10^3 ergs per mm² of ultraviolet light (phage survival, 10^{-2} to 10^{-3}), and practically no lysis corresponded to spots of more heavily treated phage. On the unsupplemented (or partially supplemented) M agar (Fig. 1), very little growth was ever observed on the plates around the cut discs, apart from a very light background growth of the vitamin-requiring mutants. The back-mutation rate was negligible in all the experiments reported. Within the discs spotted with phage, usually no growth was observed in correspondence with untreated phage, except in a few experiments at a low multiplicity of infection. Distinct colonies appeared irregularly near the edges of discs containing phage treated with 4×10^3 and sometimes 8×10^3 ergs per mm² of ultraviolet light. Evenly distributed colonies appeared on the discs spotted with phages treated with 16×10^3 and 32×10^3 (and sometimes also with 8×10^3) ergs per mm² of ultraviolet light, which give no appreciable lysis on the supplemented M agar. It might, therefore, be argued that removal of plaque-forming ability prevented the destruction of transductants by superinfection with phages added or later released by lysed cells. It is also evident that transducing ability of phage is not affected at the same rate as plaque-forming ability (Fig. 2). Only in transductions with phages treated with 32×10^3 ergs per mm² of ultraviolet light is some reduction of transducing ability often observed. The highest rate of transduction is about four times lower than that obtained with streptomycin-resistant markers on BHI agar (Table 2), but the conditions are, in many respects, not comparable. Goldschmidt and Landman (1962) found that the transducing ability of ultraviolet-treated virulent mutant phages was regularly inferior to that of a similarly treated temperate phage. The transducing ability seems not to be significantly affected by the multiplicity of infection of the total phage, in a range of multi-

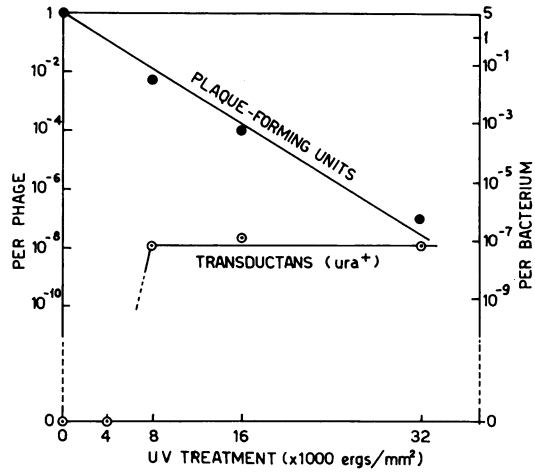

FIG. 2. *Transduction ability and survival of ultraviolet-inactivated phage 80 in Staphylococcus aureus 80. Donor: purA purD str-r; recipient: ura. Both curves refer to both ordinate parameters.*

TABLE 2. *Characters transduced by phage 80*

Character transduced	Symbol	Donor strain (symbol)	Recipient strain (symbol)	Selective medium	Highest transductant frequency (per phage)
Uracil-independence	*ura*⁺	Wild type	*ura*⁻	M*	2.1×10^{-8}
		purA purD str-r	*ura*⁻	M*	11.0×10^{-8}
Riboflavine-independence	*rib*⁺	Wild type	*rib*⁻	M*	5.0×10^{-8}
		purA purD str-r	*rib*⁻	M*	6.5×10^{-8}
Guanine-independence	*purA*⁺	*purA1 purD str-r*	*purA2*⁻	M*	0.5×10^{-8}
		Wild type	*purA2*⁻	M*	0.1×10^{-8}
Streptomycin-resistance	*str-r*	*purA purD str-r*	Wild type	BHI + St†	5.0×10^{-7}
		purA purD str-r	*ura*⁻	BHI + St†	2.0×10^{-7}

* Ultraviolet-inactivated phages.

† Streptomycin added 4 hr after plating of transduction mixture.

TABLE 3. *Transduction with ultraviolet-inactivated phage 80**

Ultraviolet (ergs/mm²)	Plaque-forming ability		Frequency of transductants		
	Nt/No	Per disc (Nt)	Per disc	Per phage particle (No)	Per cell
0	1	10^{10}	0	0	0
8,000	2×10^{-3}	2×10^{7}	120	1.2×10^{-8}	6.0×10^{-8}
16,000	3×10^{-6}	3×10^{4}	230	2.3×10^{-8}	11.5×10^{-8}
32,000	2×10^{-9}	20	115	1.2×10^{-8}	5.7×10^{-8}

* Donor strain: *purA purD str-r*. Recipient strain: *ura*. Nt = number of plaque-forming units in ultraviolet-treated lysates; No = number of plaque-forming units in untreated lysates. Phage particles (plaque-forming ability, No) = 10^{10} per disc; bacterial cells (colonies count) = 2×10^{9} per disc; multiplicity of infection = 5.

plicities from 0.1 to 5. The lytic ability of the phage is obviously higher with higher multiplicities of infection. All of the more than 100 transductants tested bred true and retained their sensitivity to phage 80.

DISCUSSION

Nutritional mutants appear to be an indispensable prerequisite for the biochemical and genetic exploration of a microbial species. They had not been isolated in *Staphylococcus* before the work of Ritz and Baldwin (1962), which was, however, confined to tryptophanless mutants. However, differential nutritional requirements among many wild isolates of *S. aureus* had been observed by Edgar and Stocker (1961) and utilized for biochemical and genetic analysis. By use of a synthetic medium similar to the M medium of Ritz and Baldwin (1962), but deprived of purines and pyrimidines, we have shown the possibility of isolating a large pattern of biochemical mutants, most of which have an impaired nucleotide biosynthesis.

The purine-requiring mutants isolated throughout this work allow some speculation about the interconversion of purine derivatives in *S. aureus*. We can assume that, as in all the microorganisms and macroorganisms studied so far, inosinmonophosphate (IMP) is the general precursor of the nucleic acid purine nucleotides, guanylic acid (GMP) and adenylic acid (AMP). As a matter of fact, all the single or double mutants requiring purine derivatives which we have obtained grow on inosine, and all but one grow on hypoxanthine. Since all the purine-requiring mutants also grow on media containing both adenine and guanine, it may be that these preformed purines can be converted (directly or indirectly) to AMP and GMP, respectively. All the single-step mutants but one, however, are able to grow on guanine alone, and this strongly suggests that the pattern of purine interconversion is such that guanine can be converted to both GMP and AMP, whereas adenine is only convertible to AMP. This is in good agreement with the observation by Wood and Steers (1955, 1959) that, in *S. aureus*, C^{14} from labeled guanine is incorporated in both GMP and AMP, whereas C^{14} from labeled adenine is only found in AMP. The guanine-requiring single mutants may be assumed to be blocked in the de novo purine biosynthesis. The two-step mutants provide further information on the pathways of nucleic acid purine nucleotide synthesis from preformed purines. From the observed pattern of mutation, we can assume that guanine is converted into IMP through a series of enzymatic reactions involving at least two successive steps. The first is a ribosilation (guanine + ribose-1-phosphate $\rightarrow$ guanosine + Pi; Kalckar, 1947), the second is a reductive deamination of the guanine residue to the hypoxanthine residue. Mager and Magasanik (1960) demonstrated in *Escherichia coli* the presence of an enzyme converting GMP to IMP, with reduced nicotinamide adenine dinucleotide phosphate as hydrogen donor. This, however, has no action on guanosine. The observed pattern of mutation in *S. aureus* is incompatible with the presence of such an enzyme acting at the nucleotide level, and requires a similar enzyme acting at the nucleoside level. Otherwise, any strain able to convert guanine to GMP should also be able to synthesize AMP through IMP. On the contrary, many double mutants grow on adenine plus guanine, and not on guanine alone. However, they grow on guanosine alone. Thus, guanosine can give rise to IMP in such mutants, whereas guanine (and therefore GMP which is formed directly from guanine)

cannot. The enzyme converting guanine to guanosine should be absent in the mutants designated *purD*, and the presumptive enzyme performing the reductive deamination of guanosine should be missing in the mutant designated *purE*. A tentative and simplified pattern of purine interconversion is shown in Fig. 3.

Two mutants do not fit in this scheme. One is a single-step mutant (*purC*) requiring both guanine and adenine, or guanosine alone. It has the phenotype of the two-step mutants *purA purD* and might be derived from a double mutation or a deletion covering two adjacent genes. Unfortunately, it has been lost. The second is a double mutant from a *purA* mutant growing with the ribosides of guanine or hypoxanthine but not with the free bases. One can put forward the hypothesis that some aspecific factor involved in purine ribosilation is missing in this mutant.

Wood and Steers (1955, 1959) isolated in *S. aureus* a chloramphenicol-resistant mutant, 1-ACR, which turned out to be purine-requiring. Its purine requirement is met by adenine together with either hypoxanthine, axanthine, or guanine. This requirement has been explained by assuming the loss of a single enzyme catalyzing a deacylation in both the de novo purine synthesis and the conversion of IMP to AMP. The mutant 1-ACR fits well in the scheme shown in Fig. 3, its metabolic blocks being in 1 and 4.

Transduction of nutritional characters within a single strain of *S. aureus*, first obtained by Ritz and Baldwin (1962) with trpytophanless mutants, widely increases the possibilities of this method of genetic analysis in the species. It has been shown to be of paramount importance in *Salmonella* (Demerec and Hartman, 1959) for the study of fundamental problems of the structural determination and regulation of enzymes. This technique will perhaps also provide methods of approach for the genetic study of virulence in *Staphylococcus*.

The use of an inactivated virulent phage as a vector in transduction also widens the possibilities of this technique, providing an easy method to dispose of virulence without seriously affecting transducing ability. The much steeper slope of the inactivation curve of the plaque-forming ability as compared with that of the transducing ability clearly shows the passive role of the carrier phage in this transduction process.

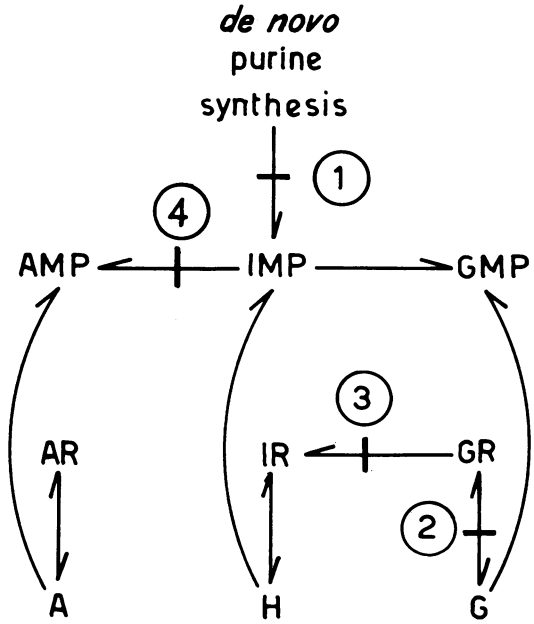

FIG. 3. *Simplified model of biosynthesis of purinic nucleotides in Staphylococcus. Abbreviations: AMP, adenilic acid; IMP, inosinic acid; GMP, guanilic acid; AR, adenosine; IR, inosine; GR, guanosine; A, adenine; H, hypoxanthine; G, guanine. Block 1, mutation purA; block 2, mutation purC block 3, mutation purE. Strain 1-ACR of Wood and Steers (1959) would be blocked in both 1 and 4.*

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