Restriction-deficient Mutants of *Staphylococcus aureus*

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SUMMARY

A series of restriction-deficient mutants was isolated from non-lysogenic strains of *Staphylococcus aureus* belonging to phage groups I and II. Some mutants were sensitive to all phages tested. With one possible exception, all the mutants were unaffected in their modification systems. The breakdown of DNA of phages, restricted in the parental strains, was reduced in both the mutants that were tested. The restriction in propagating strain 3A could be transduced to its restriction-deficient mutant. The transduction efficiency increased after ultraviolet irradiation of the transducing phage suggesting that the gene for restriction is present on the bacterial chromosome.

INTRODUCTION

The phage patterns of *Staphylococcus aureus* fall into groups I, II, III, and IV. The strains in each group are mainly sensitive to phages of the same group. As nearly all phages are adsorbed by all strains, the insensitivity of a strain to a phage is either due to lysogenic immunity or to restriction and modification. Different patterns within a group are often due to lysogenic immunity (Rountree, 1959; Blair & Carr, 1961; Winkler & Grooten, 1961; Asheshov & Winkler, 1966). Differences between groups are thought to be due to restriction and modification (Ralston & Baer, 1964*a, b, c*; van Boven *et al.*, 1974; Stobberingh, 1975). Indicator strains heated for a short time at 56 °C can often be used to differentiate between restriction and lysogenic immunity, since heating reduces restriction but does not affect immunity (Asheshov & Jevons, 1963; Ma & Mandle, 1961).

All work on restriction and modification in *S. aureus* is based on plating efficiencies, on host ranges of modified phages and on the reduction of restriction in heated bacteria. The theory is borrowed from the well-described restriction systems in *Escherichia coli* (Arber & Dussoix, 1962; Arber & Linn, 1969; Boyer, 1971; Arber, 1974). This paper describes experiments designed to produce other evidence for restriction systems in *S. aureus*.

METHODS

Media. Nutrient broth and nutrient broth agar (Difco) with CaCl₂ (876 mg l⁻¹) were used throughout.

Phage-typing was done according to Blair & Williams (1961) but the plates were incubated at 37 °C instead of 30 °C to reduce restriction. Indicator plates with heated bacteria were prepared by heating 2 ml of an overnight culture in a water bath for 2 min at 56 °C (survival 70 %) before flooding.

Coagulase (tube test) and fermentation tests were performed as described by Baird-Parker (1963). DNAase was tested on DNase test agar (BBL). Antibiotic sensitivity was determined on sensitivity agar (Difco) with Multodisks (Oxoid).

Strains. The propagating strains (PS) of the International Phage Typing System and the non-lysogenic strains 879 (group II; Winkler & Grooten, 1961) and 57 (Winkler *et al.*, 1965) were used. The cured strains 80C, derived from PS 80 (NCTC9789) (Asheshov & Winkler, 1966), and CV4 (Van der Vijver, van Es-Boon & Michel, 1975) were obtained from the appropriate authors. PS 29 and PS 71 (respectively NCTC8331 and NCTC9315) were cured to give strains 29C and 71C.

Curing. Two ml of an 18 h broth culture was incubated for 10 min at 37 °C with 1 ml of a 1:100 dilution of rabbit antiserum specific against each lysogenic phage ($K > 100$). Mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan) was then added at 0.5 µg ml⁻¹. Samples were taken after various incubation times and spread on agar plates and the colonies were tested for phage sensitivity by cross-streaking. After mitomycin induction, cured strains yielded no phage using strain 80C R3 (see below) as indicator.

Mutagenic treatment. Ethyl methanesulphonate (EMS; Koch-Light) was dissolved in a solution containing (g l⁻¹): Mohr's salt. 6H₂O, 0.028; MgSO₄. 7H₂O, 0.2; NH₄Cl, 8; KH₂PO₄, 18. This stock solution (pH 6.8) was sterilized by filtration and diluted 1:8 with distilled water before use.

An overnight broth culture (1 ml) was mixed with 1 ml of a 0.5 % (v/v) or 0.25 % (v/v) solution of EMS and incubated for 30 min at 37 °C. EMS was neutralized with 8 ml 5 % (w/v) Na₂S₂O₃; the bacteria were collected by centrifuging, washed once with broth and resuspended in 5 ml broth. The surviving population (0.01 to 0.1 %) was incubated for 5 h at 37 °C for expression.

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Koch-Light; 1 mg) was dissolved in 25 ml Tris/maleic acid buffer (6.1 g Tris and 5.8 g maleic acid l⁻¹, pH 6.0). An overnight culture (5 ml) was centrifuged and the pellet was resuspended in 2.5 ml NTG solution. After incubation for 30 min at 37 °C, NTG was removed by washing with cold broth. The bacteria were resuspended in 5 ml fresh broth and incubated for 18 h at 37 °C for expression. NTG was only used for isolating mutants of strain 879.

The presence of mutants was tested by adsorbing a restrictive phage to a sample of the population and plating this on to the parental strain. The presence of a larger number of plaques than were found in the control sample indicated the presence of r⁻m⁺ mutants able to propagate and modify the phage.

Isolation of mutants. Populations containing 40 to 60 mutants per 10⁶ survivors were plated on nutrient agar. After incubation the colonies were tested for sensitivity to the restrictive phage either by replica-plating on a plate flooded with this phage or by streaking individual colonies across a streak of the restrictive phage. Colonies showing lysis were selected as possible r⁻m⁻ or r⁻m⁺ mutants. To isolate r⁻m⁻ mutants separate colonies were streaked across adjacent streaks of the restrictive phage and the parental strain. Possible mutants were phage-typed and if they differed from the parental strain all relevant phages were titrated quantitatively on fresh and heated cells.

Phage-labelling. Five ml broth containing 2-deoxyadenosine (50 µg ml⁻¹) and [methyl-³H]-thymidine (50 mCi ml⁻¹; 80 mCi mg⁻¹) was inoculated with about 10⁸ bacteria from an overnight culture and shaken for 30 min at 37 °C. About 10⁷ (plaque-forming units) p.f.u. of phage was then added together with CaCl₂ (876 mg l⁻¹). After lysis (about 4 h at 37 °C) 0.5 ml DNAase (BDH; 1 mg ml⁻¹), 0.1 ml 0.01 M-MgCl₂ and 0.1 ml 0.01 M-Tris buffer were added and the mixture was kept at 37 °C. After centrifuging at 2000 g to remove debris, the phage was concentrated by centrifuging at 90000 g for 2 h. The sediment was taken up in 6 ml broth and kept at 4 °C. The activity was about 1 d.p.m. per 10⁴ p.f.u.

Degradation of labelled phage DNA. Labelled phage (1 ml; 3 × 10⁸ p.f.u.) was mixed with

3×10^8 bacteria (at the end of the exponential growth phase) in 1 ml nutrient broth with 0.1 ml CaCl_2 (876 mg l^{-1}) and methionine (1 mg ml^{-1}). After adsorption for 10 min at 4 °C the suspension was centrifuged at room temperature, washed with cold broth, resuspended in 2 ml broth and incubated in a water bath at 30 °C for 30 min. Samples (0.2 ml) were removed at intervals and the activity of the trichloroacetic acid (TCA)-soluble fraction was determined, as a measure of DNA breakdown (Simmon & Lederberg, 1972; Verhoef, van Boven & Holtrigter, 1972). Data are given as a percentage of the total activity (d.p.m.) of a sample taken at zero time (without TCA). Activity was determined by adding each sample to 10 ml of counting fluid [toluene (750 ml) plus Triton X-100 (250 ml) containing 500 mg 2,5-diphenyloxazole and 50 mg 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] and counting in a Nuclear Chicago mark II scintillation counter.

Transduction was performed as described by Asheshov (1966). Phage (10^{10} p.f.u.) was added to 9 ml of an overnight culture [10^8 colony-forming units ml^{-1}] in broth with CaCl_2 (200 mg l^{-1}). The mixture was incubated for 15 min at 37 °C and centrifuged at 2000 g. The sediment was resuspended in 20 ml nutrient broth containing 0.01 M-sodium citrate and incubated for 3 h at 37 °C. After centrifugation and washing with broth the sediment was incubated with 3 to 5 ml of restrictive phage (multiplicity of infection about 10) for 30 min at 37 °C and plated. A control culture without transducing phage was treated in the same way. All colonies were tested for sensitivity against the selecting phage and possible transductants were phage-typed.

RESULTS

Cured and non-lysogenic strains

For genetic studies on restriction systems, parental strains without lysogenic immunity are preferable. The phage patterns of the non-lysogenic strain 879 and the cured strains 80C, CV4 and 29C are given in Fig. 1 together with the patterns of the strains from which they are derived. The phage pattern of the non-lysogenic PS 3A (NCTC8319) is shown in Fig. 2.

The cured strains of group I (29C, CV4 and 80C) are sensitive to all group I phages but are nsensitive to many group II and III phages.

Restriction-deficient mutants

A number of the strains in Fig. 1 were treated with mutagens and the resulting populations were tested for colonies sensitive to a restricted phage (selecting phage). Most such possible mutants proved to be unstable and reverted to the parental pattern on subcultivation. About 1500 colonies had to be tested in each experiment to isolate a mutant that was stable on subcultivation. None of the isolated mutants (Table 1) differed from the parent strain with regard to their general properties, such as fermentation, DNAase production, coagulase production and antibiotic resistance.

The phage patterns established by quantitative phage titrations of some mutants are shown in Fig. 2. PS 3A is a non-lysogenic group II strain, restrictive for all group I and III phages. The mutant 3A R1 had become sensitive to all phages tested. It appeared to be the result of a one-step mutation, and this was confirmed by a back mutation rate of 4×10^{-8} from 3A R1 to 3A. This suggests that one *rm* system (*rm_{II}*) is responsible for the insensitivity of PS 3A to phages of groups I and III.

Strain 879, also a non-lysogenic group II strain, is insensitive to many group I and III phages. It also shows a weak (10^{-2}) restriction for phage 3A as detected by heating. Several mutants were isolated from this strain. Mutant 879 R1 (selected with phage 3A) is a one-step

Fig. 1. Quantitative phage patterns of non-lysogenic and cured strains and their parents. Black columns represent the $\log_{10}$ of the phage titre on each strain ($\log_{10}$ of the phage titre on the PS = 8). White columns represent titres on heated bacteria of each strain.

Table 1. *Isolated mutants*

Strain	Selecting phage	Independent isolations	Mutant	Phenotype (hypothetical)
3A	80/80C	4	3A R1	$r_{3A}^+ m_{3A}^+ r_{II}^- m_{II}^+$
879	3A	9	879 R1	$r_{879}^- m_{879}^+ r_{II}^+ m_{II}^+$
879	80/80C	4	879 R2	$r_{879}^+ m_{879}^+ r_{II}^- m_{II}^+$
879 R2	6	1	879 R3	$r_{879}^- m_{879}^+ r_{II}^- m_{II}^+$
80C	54	3	80C R1	$r_{80A}^+ m_{80A}^+ r_{80B}^+ m_{80B}^+$
80C R1	3A	1	80C R2	$r_{80A}^- m_{80A}^- r_{80B}^+ m_{80B}^+$
80C R2	71	1	80C R3	$r_{80A}^- m_{80A}^- r_{80B}^- m_{80B}^+$
CV4	54	4	CV4 R1	

mutant, lacking the restriction for phage 3A and with reduced restriction for some group I phages. Mutant 879 R2, selected with phage 80/80C, is a separate one-step mutant with reduced restriction for many phages in groups I and III, but still restrictive for phage 3A. Mutant 879 R3, isolated from strain 879 R2 with phage 6 as selecting phage, appears to be a double mutant sensitive to most group I and III phages with the exception of phages 75, 77, 77A and 85.

After mutagenic treatment of strain 80C, and with phage 54 as the selecting phage, the mutant 80C R1 was obtained. This is more sensitive to group III phages, but the sensitivity still increases after heating. Subsequent treatment of strain 80C R1 and selection with phage 3A resulted in mutant 80C R2, which had lost the restriction for all group III phages (= rm_{80A}) as shown by the similar sensitivity for these phages before and after heating. The restriction for group II phages (= rm_{80B}) still existed. This rm_{80B} system was eliminated in mutant 80C R3, obtained after mutagenic treatment of strain 80C R2 and selection with phage 71. This mutant is sensitive to all group I, II and III phages.

Four independent mutants (CV4 R1) with the same pattern were obtained from strain CV4.

Fig. 2. Quantitative phage patterns of r^- mutants. For details see legend to Fig. 1.

Fig. 3. Breakdown of $[^3\text{H}]$ thymidine-labelled phage DNA in various strains. (a) Phage 80/80C in the restrictive strains 879 (○) and 3A (●), the restriction-deficient mutant 3A R1 (▲), in the group I strain 29 (Δ) and its cured derivative 29C (□), and in the non-restrictive strain 80C (■). (b) Phage 3A in the restrictive strain 80C (○), its restriction-deficient mutant 80C R3 (●), and PS 3A (▲). (c) Phage 6 in the restrictive strains PS 53 (○) and 80C (▲), in the restriction-deficient mutant 80C R3 (●), and in PS 6 (■). Results show the radioactivity (d.p.m.) in the TCA-soluble fraction as a percentage of the total activity in a reference sample (no TCA extraction) at zero time. The degradation in restrictive samples at time zero is probably a result of centrifugation at room temperature.

Fig. 4. Modification by mutants. Quantitative phage patterns of various phages propagated on parental strains and restriction-deficient mutants. For details, see legend to Fig. 1.

The restriction for group III phages was reduced in these mutants. The restriction for group II phages still existed. Attempts to eliminate this rm system were not successful.

Degradation of labelled phage DNA

Figure 3(a) shows the breakdown of phage 80 propagated on strain 80C in the restrictive strains 879 and 3A. Breakdown in the restriction-deficient mutant 3A R1 and in the group I strain 29C was much less. In strain 80C less than 15 % of the DNA appeared in the soluble fraction. Figure 3(b) shows that the DNA of labelled phage 3A was relatively stable in PS 3A, but quickly broken down in strain 80C and to a lesser extent in the mutant 80C R3. Breakdown of the DNA of phage 3A was also observed in the restrictive strains 57, 879 and PS 6. Phage 71 was broken down (30 %) in the restrictive strain 3A but not (6 %) in the

permissive strain 879. Figure 3(c) shows the breakdown of phage 6 in the restrictive strains 80C and 53 and the reduced breakdown in mutant 80C R₃.

Analogous experiments with restrictive strains after heating always confirmed that DNA breakdown was reduced in heated strains.

It should be noted that a restriction measured by phage titre (efficiency of plating) as 10^{-3} or 10^{-6} means that 1 in 1000 or 1 in 10^6 phage particles escape. In both cases over 99 % of the phage DNA is broken down. A quantitative relationship between restriction and DNA breakdown cannot therefore be expected. Furthermore the TCA-soluble DNA represents the effect of exonucleases on the large DNA pieces presumably produced by the restriction endonuclease and is not a direct measure of restriction.

Modification by mutants

In *E. coli* about 50 % of the isolated r^- mutants are also m^- (Glover, 1970; Wood, 1966). The modification behaviour of our mutants was studied by comparing the host range of various phages after propagation on parent and mutant strains (Fig. 4). The reversal patterns after re-propagation on the original PS have not been included in Fig. 4. The sensitivity of the non-lysogenic strains, shown on the left, is the best parameter for modification of a phage. Phage 80 propagated on strains 80C R₂ or 80C R₃ retains approximately its original host range, suggesting that the mutants are m^+ . Phage 80 causes plaques on heated cells of PS 3A (Fig. 2) and can then be propagated on PS 3A. Figure 4 shows that phage 80/3A is modified to a phage with a group II pattern. Propagation on strain 3A R₁ produces a comparable modification. Strain 879 and its mutants also modify phage 80 to phages with a group II pattern.

Phage 3A propagated on strain 879 or its mutants receives the 879 modification, with restriction in PS 3A.

The modification behaviour of strain 80C R₂ (and 80C R₃) was not the same for all phages (Stobberingh, 1975). Phage 80/80C keeps its group I pattern after propagation on these mutants (Fig. 4, line 2). The same was true for phage 29 (group I). Phage 47 (group III) also had a group I pattern and had lost several reactions in group III after propagation on strains 80C, 80C R₁, 80C R₂ or 80C R₃ (Fig. 4, lines 12 to 14). In contrast, phage 3A (Fig. 4, lines 10 and 11) though modified by strain 80C to a group I pattern, did not obtain this modification in the mutants, although it lost its group II modification. The same was true for phages 3C and 71. This suggests that the modification system rm_{80A} is altered. With this exception, all the isolated mutants were r^-m^+ . All attempts to isolate m^- mutants either from r^- mutants or from parental strains were unsuccessful.

Transduction of rm_{3A} to mutant 3A R₁

In other genera, genes coding for *rm* systems have been found on the chromosome, on plasmids and on lysogenic phages. Asheshov & Rippon (1959) showed the presence of a restriction system on the lysogenic phage F in PS 80. It was important to know the localization of an *rm* system affecting a whole phage group. Thus a transduction experiment was performed with phage 80/3A as a transducing phage and the mutant strain 3A R₁ as recipient. After expression, the population was infected with phage 6 as the selecting phage and the infected bacteria were plated. As all parental bacteria would be killed by the selecting phage, the survivors would either be transductants or bacteria which had escaped infection. All growing colonies were tested for sensitivity to the selecting phage and possible transductants were phage-typed. Control plates from experiments without transducing phage were mainly negative, thus excluding back mutations.

Fig. 5. Transduction of restriction from PS 3A to restriction-deficient mutant 3A R1, with phage 80/3A as transducing phage and phage 6 as selecting phage. The transducing phage was irradiated with ultraviolet light on a mechanical shaker. ○, Phage survival; ●, transduction frequency.

Experiments with ultraviolet-irradiated transducing phage gave the results shown in Fig. 5. The transduction frequency of 10^{-5} increased to 10^{-4} after 2 min of ultraviolet irradiation. This character is generally present in transduction of chromosomal markers (Arber, 1960).

DISCUSSION

The groups of strains of *S. aureus* with related phage patterns are thought to be restriction groups carrying different *rm* systems. This conception is supported by the phage patterns of the cured strains (Fig. 1) and particularly by the patterns of the isolated mutants (Fig. 2). The mutants generally gained sensitivity for a whole group of phages in one step. It is also interesting that so many independent mutants had the same phage pattern (Table 1).

Mutant 3A R1 appears to be a one-step mutant, both from its origin and its back mutation rate. The reduced breakdown of phage DNA in this mutant (Fig. 3a) shows that it is deficient in restriction. The frequency of transduction of restriction from PS 3A to strain 3A R1 is rather high but the increase of transduction frequency after ultraviolet irradiation of the transducing phage is consistent with a chromosomal localization of the gene coding for the presumed restriction enzyme. Sussenbach, Monfoort & Stobberingh (1976) isolated and characterized a restriction enzyme from strain 3A, which broke the DNA of phage 6 in many pieces, but did not affect the DNA of phage 3A. The enzyme did not require *S*-adenylmethionine or ATP. No such enzyme activity was detectable in extracts of strain 3A R1.

For the other mutants the picture is less complete. The different mutants of strains 879 and 80c certainly carry different mutations and 80c R3 and 879 R3 are presumably double mutants. All mutants tested were deficient in the breakdown of phage DNA (Fig. 3) but the experiments with labelled phage cannot differentiate between strong and weak restriction. The different mutations in mutant 80c R3 might be mutations in different enzymes (as suggested in Table 1, last column) or different mutation of the same enzyme. In the latter case, the mutation should affect the recognition site or the affinity of the enzyme as the activity against phages of other groups is not affected.

With one possible exception, no $r^{-}m^{-}$ mutants were obtained by us or by Dr E. H. Asheshov (personal communication). This lack of $r^{-}m^{-}$ mutants makes it unlikely that the rm systems in *S. aureus* are governed by a three-gene system as are those of type I in *E. coli*. Together with the properties of the enzyme isolated from PS 3A, it seems more likely that the rm systems are of type II.

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