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Bacteriophage 604: a marker phage for multi-resistant *Staphylococcus aureus* in Australia

B. INGLIS, I. HEDING*, M. MERRYLEES AND P. R. STEWART†

Department of Biochemistry, Faculty of Science, Australian National University, Canberra, ACT 2601, Australia

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SUMMARY

Of 28 multi-resistant isolates of *Staphylococcus aureus* collected during 1986 from hospitals in major cities around Australia, 27 were found to contain the same prophage (denoted phage 604). Hospital isolates carrying three or fewer resistance markers, and community isolates carrying one or no resistance markers, did not carry this prophage. Phage 604 does not confer antibiotic resistance on its lysogens, nor does it increase virulence in chick embryo assays. Phage 604 appears to be a correlate of antibiotic multi-resistance in *S. aureus* in Australia, and may provide a molecular marker for incipiently epidemic strains of this bacterium in Australian hospitals.

INTRODUCTION

Specific epidemiological markers for virulent staphylococci are not well characterized. The production of a range of toxins, exoenzymes, and anti-immunity factors has been described, and various roles ascribed to these in determining the virulence of particular strains [1, for review]. However, the possession of these 'virulence factors' does not always correlate with the ability of particular strains to cause hospital outbreaks.

In *Staphylococcus aureus*, the association between multiple resistance to antibiotics and virulence has received close attention [2], though with no apparent conclusion. With the view that this association might usefully be tested using an epidemiological approach, we described previously the lysogenic carriage of a bacteriophage (denoted phage 604) by multi-resistant strains of *S. aureus* (MRSA) isolated since 1980 from hospitals in Australian cities [3]. We report here an extension of this analysis to further multi-resistant isolates of *S. aureus*, to predominantly antibiotic-sensitive isolates from hospital patients, and to antibiotic-sensitive isolates from healthy, non-hospitalized humans.

* Present address: Department of Biochemistry, Imperial College of Science and Technology, Imperial College Road, London SW7 2AZ.
† For correspondence and reprint requests.

Table 1. *Origin of Australian isolates analysed for phage 604*

City	Community isolates	Clinical isolates* 4–8	Clinical isolates* 0–3
Adelaide	—	4	8
Canberra	23	9	17
Darwin	—	2	4
Hobart	—	4	3
Melbourne	—	2	2
Perth	—	2	4
Sydney	—	5	5
Total	23	28	43

The community strains were isolated from healthy students in Canberra. The clinical isolates were obtained from different patients in a major hospital in each city.

* The ranges refer to the number of resistance markers carried. Community strains carried one or no markers.

METHODS

Bacterial strains and phages

Clinical isolates were collected from major hospitals in seven state and territory capitals of Australia during 1986 (Table 1). This collection consisted of 71 isolates of clinical origin, tested for resistance to eight antibiotics: benzylpenicillin (Pc), methicillin (Mc), tetracycline (Tc), chloramphenicol (Cm), streptomycin (Sm), kanamycin (Km), erythromycin (Er) and sulphathiazole (Su). Forty-three of these were resistant to three or fewer antibiotics (none resistant to methicillin), and 28 were resistant to four or more antibiotics (all but one methicillin-resistant). Phage typing of a sample of 19 of these strains showed no consistent pattern of types, as reported previously [3].

A collection of 23 community isolates of *S. aureus* was obtained from healthy students (not administered antibiotics for 3 months or more before the time of sampling) from three different Canberra educational institutions. Fourteen of these were resistant to benzylpenicillin but not to any of the other antibiotics tested.

Two strains on which phage 604 grows well, and forms lysogens, were also used: PS6 (propagating strain for phage 6 in the international set) and S1030 (general-purpose phage-propagating strain). These, and MRSA isolates carrying phage C [4], were obtained from Alison Vickery, Royal Prince Alfred Hospital, Sydney. Phage 604 was obtained by mitomycin C induction of MRSA strain SK604 as described previously [3].

Construction of lysogens

Phage 604 lysogens were prepared by spotting a phage 604 suspension on to a lawn of the phage-sensitive cells, and after overnight growth, plating samples from the lysed area for surviving cells. Selected clones of these survivors were shown to carry prophage 604 by their resistance to infection by phage 604, and by their ability to produce a phage with the same host range as phage 604 when induced with mitomycin C [3].

Chick embryo virulence assay

The method used was essentially that of McCabe [5]. Fertilized eggs from White Leghorn or Rhode Island Red fowl were obtained from a domestic supplier. Groups of 10 eggs were inoculated intra-allantoically with 0·1 ml of a saline-washed suspension of bacterial cells (6×10^8 cells/ml) prepared from an overnight culture grown in trypticase soy broth. Control sets of eggs were mock-infected using saline. Eggs were incubated at 37 °C with 60% relative humidity, rolled twice a day, and candled daily to determine viability. The assay is sensitive to the age of the embryonated eggs: using 6-day eggs, all were killed within the first day of inoculation with 6×10^7 cells of SK604, whereas for 7-day eggs, a 50% kill normally occurred on the third day, and 10-day eggs were not killed at all 4 days after inoculation. Seven-day eggs were used in these assays.

Survival curves of inoculated eggs were analysed using a proportional hazards model to generate an estimate (p_{ij}) of an embryo dying on a particular day [6]. Virulence coefficients are calculated as values of p_{ij} standardized to a reference value of 0·00 for strain SK604, the type MRSA carrying phage 604. Positive and negative values of the virulence coefficient indicate greater and lesser virulence than SK604 respectively.

Other methods

Methods for the induction and plating of phage, preparation of cellular DNA, and probing for prophage DNA have been described previously [3].

RESULTS

Presence of phage 604 in clinical and community isolates

Cellular DNA from the isolates was digested with endonuclease, fractionated by electrophoresis, and probed with labelled phage 604 DNA to detect prophage sequences, related or identical to phage 604, in the cells. The number of isolates of *S. aureus* examined for phage 604 is shown in Table 1. Fig. 1 shows prophage fragments hybridizing with phage 604 DNA in a selection of the isolates used in this study. None of the 23 community isolates examined (representative examples are shown in tracks 2–6) contained phage 604 as such, though the presence in most cases of DNA fragments of different size to those found in phage 604 but which hybridized to differing degrees with the phage 604 probe indicates that many of these isolates contain phages related to 604 (e.g. tracks 4–6). In other cases the sequence similarity is low, but sufficient nevertheless to reveal fragments belonging presumably to other prophages (e.g. track 3). Three community isolates (not shown) carried sequences which hybridized to phage 604 to an extent which was barely detectable. These trace similarities resemble those seen in the most distantly related members of the international typing set [7].

In the Australian clinical isolates (tracks 7–18), the presence of prophage 604 appears to be related to the number of resistance determinants carried by the cell. Thus, 27 of 28 isolates carrying resistance markers for four or more antibiotics contained prophage 604 (tracks 7–12). All 43 clinical isolates with three or fewer resistance determinants (tracks 13–18) had no prophage 604, but like the

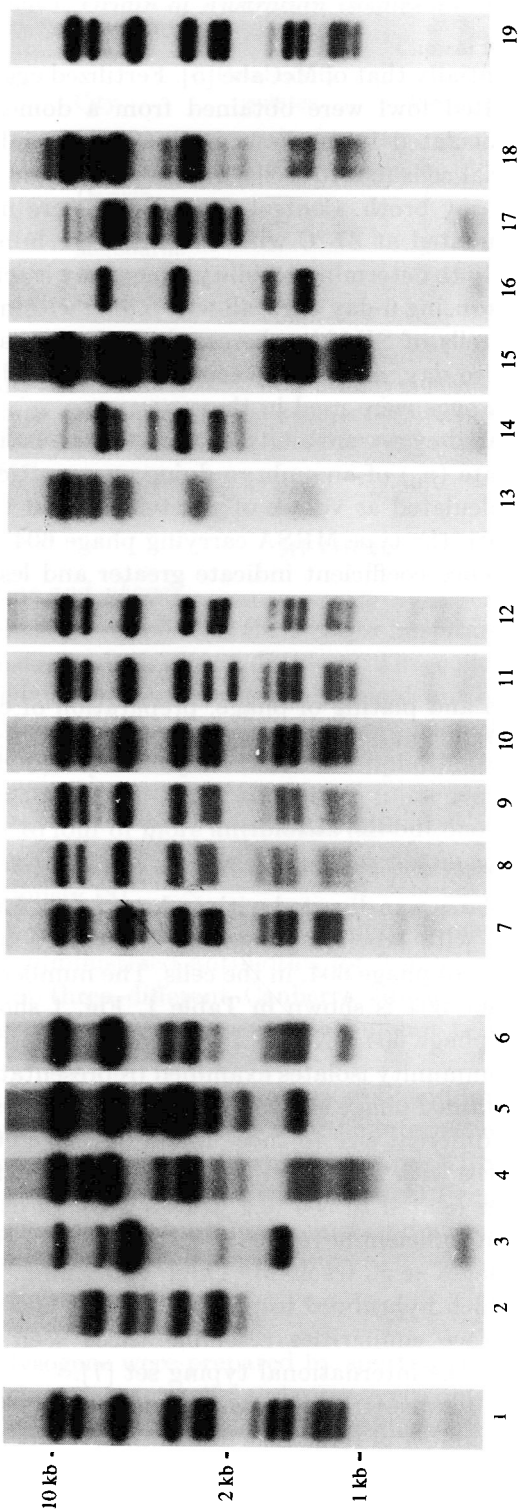

Fig. 1. Chromosomal DNA (1 μg) of representative community isolates (tracks 2–6), and clinical isolates of Australian origin resistant to 4–8 antibiotics (tracks 7–12) or to 0–3 antibiotics (tracks 13–18), was digested with *Hind* III, electrophoresed, hybridized with phage 604 [^{32}P]DNA, and the gel autoradiographed. The figure is a composite prepared from a number of gels, and accordingly there are slight variations in the mobility of DNA fragments; phage 604 digests were run with the cellular DNAs in each experiment. Tracks 1 and 19 show the relative fragment sizes and hybridization intensities for SK604 DNA digested with *Hind* III; SK604 is the *S. aureus* strain from which phage 604 was originally isolated [3].

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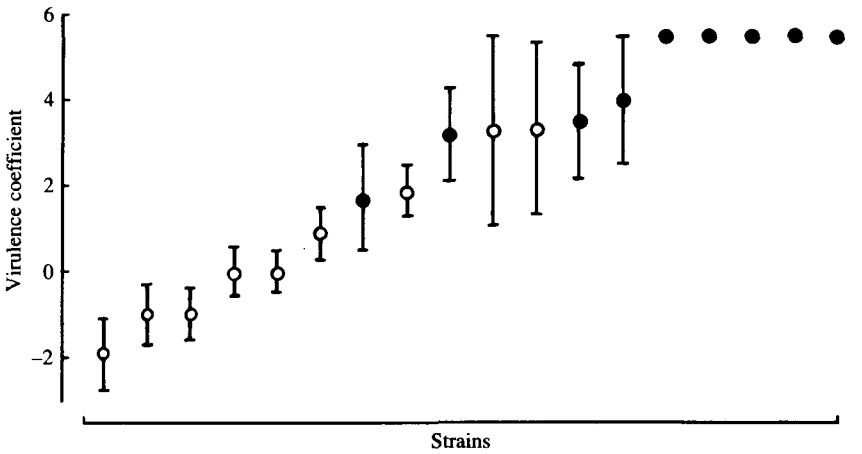

Fig. 2. Virulence coefficients for community (○) and MRSA (●) isolates of *S. aureus*. Each isolate was assayed in 10 embryonated eggs (7 days post-fertilization), inoculated at 6×10^7 cells per egg, and the killing curve analysed to generate virulence coefficients relative to strain SK 604 (coefficient of 0·0) as described in the Methods section. Bars indicate standard deviations; the five highest values of virulence coefficient are maximal values assayable with 7-day-old eggs, and standard deviation values are not shown because they are not meaningful. The mean virulence coefficient (±S.D.) for community isolates as a group was 4·4±1·4 ($n = 9$), and for MRSA isolates 0·6±1·9 ($n = 9$).

community isolates, many yielded DNA fragments of different size but hybridizable with phage 604 fragments and thus showing certain sequence similarities to phage 604.

Effects of lysogenization with phage 604 on resistance to antibiotics

Four different host strains were selected for lysogenization with phage 604: the standard laboratory strains PS6 and S1030; L17, a representative community isolate carrying resistance to benzylpenicillin; and clinical isolate 186, also carrying resistance only to benzylpenicillin. Prior to lysogenization, none of these strains carried sequences in their cell DNA corresponding to phage 604. When the constructed lysogens were tested for antibiotic resistance, none showed any change compared with the non-lysogenized host.

Effect of lysogenization with phage 604 on virulence of community and MRSA isolates

Nine community isolates and nine multi-resistant clinical isolates carrying phage 604 were examined for virulence towards 7-day-old fertilized chick eggs (Fig. 2.). Community isolates are concentrated towards the right end of this plot, and clinical isolates to the left, suggesting that the former are generally more virulent than the latter in this assay. However, the mean values of the virulence coefficients for the two groups are not statistically different, and further experiments would be required to test the trend seen here. Nevertheless, these results imply that neither lysogenic carriage of phage 604 nor multi-resistance, the two prominent phenotypic differences between the two groups, is associated with an increase in virulence in MRSA strains.

Table 2. *Virulence of phage 604 lysogens and non-lysogens*

Bacterial strain	Virulence coefficient
PS6	$-0{\cdot}7 \pm 0{\cdot}7$
PS6 (604)	$-1{\cdot}0 \pm 0{\cdot}7$
S1030	$2{\cdot}8 \pm 0{\cdot}8$
S1030 (604)	$2{\cdot}4 \pm 0{\cdot}6$
L17	$3{\cdot}5 \pm 1{\cdot}3$
L17 (604)	$3{\cdot}6 \pm 1{\cdot}2$
L23	$2{\cdot}9 \pm 0{\cdot}7$
L23 (604)	$2{\cdot}9 \pm 0{\cdot}7$

Values given are mean virulence coefficients ± S.D. for survival assays using 10 embryonated eggs (7 days post-fertilization) and an inoculum of 6×10^7 bacterial cells per egg.

PS6 and S1030 are laboratory strains described in the Methods section; strains L17 and L23 are representative community strains. Phage 604 lysogens of these were generated and verified as such as described in the Methods section.

Likewise, when virulence was measured for four laboratory or community strains which do not normally carry phage 604, and compared with that of their counterparts lysogenized with phage 604, there was no significant difference in the virulence coefficients for the pairs of strains which could be accounted for by the phage (Table 2).

Attempts to cure MRSA strains of phage 604 were not continued after it was found that relatively high doses of acriflavin or u.v. irradiation (to give 98% kill of original cells) were required to generate non-lysogenized strains. Under these conditions, extensive rearrangement of the chromosome may occur (Inglis & Stewart, unpublished), and the consequent multiple genetic change does not allow an unambiguous interpretation of virulence differences between variants.

DISCUSSION

The association of phage 604 with multi-resistant *S. aureus*, but not with isolates carrying three or fewer resistance markers (whether of community or clinical origin), might be explained in a number of ways.

First, phage 604 itself may carry multiple antibiotic resistance genes. The experiments describing the lysogenization of laboratory strains and clinical and community isolates with phage 604, producing no concomitant change in antibiotic resistance in the lysogens, do not support this notion.

Secondly, the phage may insert as a prophage and interact with chromosomal or plasmid clusters of cryptic resistance genes, switch them on, and bring these genes under common regulation. The lysogenization experiments are likewise not consistent with this view, although the possibility is not ruled out entirely.

Thirdly, phage 604 may promote virulence of the cells which it lysogenizes, making such lysogens more likely to be found in clinical environments. The comparison of community and MRSA isolates for virulence in embryonated chick eggs does not support this view, though virulence in chick embryos may not correspond to virulence in humans. It is also noteworthy that the trend to greater virulence among non-resistant community isolates supports a prevalent though

anecdotal view that MRSA strains are not highly virulent, but because of their multiple antibiotic resistance flourish in patients with weakened or compromised defences who are taking antibiotics.

Fourthly, phage 604 may have been present originally in a progenitor clone which has given rise to the multi-resistant types now prevalent in Australian hospitals; phage 604 would thus be a clonal marker for these clinical isolates, but play no necessary part in determining their virulence or resistance. This idea is an extension of the proposal that MRSA isolates from different locations may have originated from a single clone [8]. The sudden appearance and persistence of MRSA isolates carrying a phage denoted C in a major Sydney hospital since 1974 [4] is consistent with this idea. These lysogens replaced others carrying temperate phages of mixed type which had been present since 1965, when MRSA was first detected in this hospital. The appearance of MRSA strains carrying phage C foreshadowed a new epidemic of this organism, beginning about 1976. We have found that phage C and phage 604 are identical by the criteria of endonuclease fragment sizes and hybridization analysis.

The fifth possibility is that phage 604 (as prophage) may make cells more effective recipients of resistance genes delivered by gene vectors such as other phages or conjugative plasmids, i.e. that phage 604 is a 'mediator' in the conjugative exchange of plasmid-borne resistance genes between cells [10]. We have been unable to demonstrate any greater propensity for plasmid transfer using lysogens carrying phage 604, whether acting as donors or recipients in mixed cell matings (Banyer & Stewart, unpublished).

Despite our being unable to identify a specific role for phage 604 in the epidemiology of MRSA, its presence in multi-resistant clinical isolates and its absence from isolates which carry few or no resistance genes (whether of clinical or community origin), indicate that it could be used as an epidemiological marker for established or incipiently epidemic MRSA types in Australian hospitals. To be able to detect an epidemic strain early in its proliferation may provide the means to forestall, or the time to deal with, new epidemics of MRSA.

The association of phage 604 with Australian strains of MRSA raises the question of whether the same or analogous phages might exist in MRSA populations in other countries. In a preliminary analysis we examined six MRSA strains, isolated independently from two physically distinct Boston teaching hospitals. None of these strains carried phage 604, though each appeared to carry a prophage, identical across the six strains, with DNA sequences showing partial sequence similarity (by filter hybridization) to phage 604. Perhaps these American strains harbour another prophage, with similar associations with MRSA as phage 604 has in Australia.

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