Phage typing of *Staphylococcus saprophyticus*

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SUMMARY

This study included 502 staphylococcus strains; *Staphylococcus saprophyticus*
(297 strains) *S. cohnii* (47), *S. xylosus* (10), *S. epidermidis* (67) and *S. aureus* (81).
Mitomycin C induction was performed on 100 isolates of *S. saprophyticus* and all
induced strains were reacted with each other. Twenty-six strains proved to be
lysogenic. Phages were propagated and titrated. With 12 of the phages there were
three frequent associations, named lytic groups A, B and C, which included 75 %
of all typable strains. Typability of the system was 45 % and reproducibility was
between 94·2 % and 100 %. Phages did not lyse *S. aureus* and *S. epidermidis*
strains, but they lysed *S. saprophyticus* and only rare strains of other novobiocin
resistant species. Effective *S. saprophyticus* typing serves ecological purposes and
tracing the origin of urinary strains from the skin or mucous membranes. Phage
typing in association with plasmid profiling previously described, are anticipated
as complementary methods with strong discriminatory power for differentiating
among *S. saprophyticus* strains.

INTRODUCTION

The role of *Staphylococcus saprophyticus* in human disease dates from 1961 [1, 2]
when it was recognized as the cause of urinary tract infection (UTI) in young
women. Soon confirmed in Great Britain [3, 4] it was also reported from elsewhere
[5, 6]. The species is now considered, after *Escherichia coli*, the second most
frequent agent of UTI in young women [5–7].

The main phage typing studies with coagulase-negative staphylococci were
carried out chiefly on *S. epidermidis* by workers in Great Britain [8–10], The
Netherlands [11], Germany [12, 13], and USA [14]. Phage typing studies on
S. saprophyticus are almost non-existent. De Saxe and Notley [9] analysed 29
S. saprophyticus strains and found that only two phages could lyse 3·4 % of them.
Fournet and colleagues [15] studied 24 *S. saprophyticus* strains and obtained seven
phages after mitomycin C induction.

In this paper we report the results of the study of lysogeny of 100 *S.
saprophyticus* strains. A phage set for the typing of *S. saprophyticus* is presented,
its evaluation is analysed and the results of phage typing of 297 *S. saprophyticus*
strains plus 205 strains of other *Staphylococcus* species are reported.

Bacterial strains

A group of 502 staphylococcus strains was included in this study. With two exceptions all were isolated from different individuals and were epidemiologically unrelated. Two laboratory *S. aureus* strains, kindly provided by Dr J. Naidoo (UK), were included as indicator strains. Two hundred and seven strains of *S. saprophyticus* were isolated from outpatient women with UTI and 90 strains of the same species were isolated from skin and mucous membranes of children and healthy adults. Other species were included for comparison as follows: *S. cohnii* 47 strains from skin and mucous membranes, *S. xylosus* 10 strains from skin and mucous membranes, *S. epidermidis* 67 strains from skin and pathological specimens and 79 *S. aureus* strains from pathological specimens. *S. aureus* strains W57 and 80CR5Rif were indicator strains. All strains were identified as described previously [16].

Induction of S. saprophyticus *strains*

A group of 100 *S. saprophyticus* strains (named S1 to S100, 84 from urine and 16 from skin and mucous membranes) was induced by mitomycin C following a method adapted from De Saxe and Notley [9]. Each strain to be tested was inoculated into 3 ml volumes of nutrient broth (Oxoid Nutrient Broth No. 2 2% w/v, sodium chloride 0·5% w/v) and grown overnight at 37 °C. 0·1 ml of a stock solution of mitomycin C was added to 1·9 ml of the culture in broth to give a final concentration of 0·5 μg ml^{-1}. Incubation was continued for 30 min in the water bath at 37 °C and the tubes were centrifuged for 20 min at 3500 rev./min. Supernatants were removed and discarded and the cells were resuspended in 2 ml volumes of nutrient broth containing calcium chloride to a final concentration of 400 μg ml^{-1}. These cultures were incubated for a further 2 h at 37 °C then centrifuged for 20 min at 3500 rev./min. Finally, 1·5 ml of the supernatants were collected and used for testing. After induction 10 μl of each supernatant were tested on all 100 *S. saprophyticus* strains.

Propagation and titration of phages

All phages recovered by induction were tested on lawns of the parent strains (cross-spotting). Phage reactions were recorded as follows: $\pm$, 1–19 plaques; +, 20–50 plaques; + +, > 50 plaques to semi-confluent lysis; CL, confluent lysis. Inhibition reactions were not considered as positive reactions in this study. A strain showing strong lysis was selected as propagating strain (PS) for each phage.

Phages were propagated by a soft-agar method [17] as follows: tubes containing 12·5 ml of soft agar (Oxoid Nutrient Broth No. 2 2% w/v, Oxoid Agar No. 1 0·4% w/v) were melted and cooled to 45–46 °C. To each tube was added 1 ml of a stock solution of calcium chloride to give a final concentration of 400 μg ml^{-1}, 1 ml of the supernatant of the induced strain and 0·5 ml of an overnight broth culture of the propagating strain. The agar mixture was poured on the surface of nutrient agar plate (Oxoid Nutrient Broth No. 2 2% w/v, Oxoid Agar No. 1 0·7% w/v). After overnight incubation at 37 °C 5 ml of broth were added to each plate and the soft-

agar layer was scraped off with a sterile glass rod into a tube which was shaken vigorously for a few minutes to break up the agar and extract the phage. The mixture was centrifuged at 2000 rev./min for 15 min and the supernatants were transferred to another tube and centrifuged again at 3500 rev./min for 20 min. Supernatants were finally recovered, filtered through membrane filters and titrated.

Titration of phage filtrates was carried out as described for *S. aureus* [18]: The propagating strain was inoculated into broth and incubated at 37 °C overnight. A plate of nutrient agar was flooded with the broth and allowed to dry. Tenfold dilutions of the phage suspension were made and one 20 μl drop of each was placed on the surface of the plate. After the drops were absorbed, the plates were incubated at 30 °C and examined the following day for lysis. The titre of the phage suspension was defined as the dilution that produced just less than confluent lysis.

The phages were given the same designation as the respective donor strain preceded by the character ϕ. Concentrated suspensions of all phages (ranging from $RTD \times 10^6$ to $RTD \times 10^9$) were obtained after one to five propagations of each phage. Concentrated phages were filtered through membrane filters and stored at 4 °C.

Staphylococcus saprophyticus *phage set* and phage typing*

The following 26 phages were used at $100 \times RTD$: $\phi S3$, $\phi S14$, $\phi S18$, $\phi S20$, $\phi S22$, $\phi S23$, $\phi S29$, $\phi S38$, $\phi S40$, $\phi S41$, $\phi S44$, $\phi S46$, $\phi S48$, $\phi S50$, $\phi S60$, $\phi S61$, $\phi S63$, $\phi S68$, $\phi S69$, $\phi S72$, $\phi S83$, $\phi S85$, $\phi S88$, $\phi S90$, $\phi S93$ and $\phi S97$. Phage typing methodology and recording of results were carried out as described for *S. aureus* [18]. All 502 previously mentioned strains were typed with these phages.

Evaluation of the typing system for S. saprophyticus

The typing system was evaluated in terms of typability, reproducibility and discrimination as described previously [19]. All 297 *S. saprophyticus* strains were considered in this evaluation.

Strains were considered typable if they showed at least one strong (> 50 plaques) reaction. The typability was expressed as the percentage of strains that were typable with this set of phages.

The reproducibility of the lytic reactions was evaluated by repeat testing of 12 selected *S. saprophyticus* strains showing different lytic reactions. One hundred and twenty reactions of each phage were evaluated. Discrepant reactions were considered whenever a weak reaction ($\pm$ or $+$) turned into a strong reaction ($+ +$ or CL) and vice versa. The percentage reproducibility of the lytic reactions was calculated according to the number of identical (non-discrepant) reactions obtained with each phage in 120 tests.

The ability of the phage set to discriminate between strains was assessed from the comparison of the number of major reaction differences between 100 artificially constructed random pairs from independent strains.

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* These phages are available as communicated to the phage-typing subcommittee in the Osaka Meeting (1990).

Serological relationships among phages

Sera were prepared in rabbits with the phages ϕS3, ϕS50, ϕS61, ϕS68, ϕS72 and ϕS85. Each animal was inoculated with increasing volumes of concentrated phage (10^8 p.f.u. per ml) starting with 0·5 ml, three times a week for 3 weeks. The rabbits were bled 1 week after the last injection.

To demonstrate neutralization, 0·1 ml of serum diluted 1:20 with 0·9% NaCl was mixed with 0·1 ml of phage at RTD, and incubated for 5 min at 37 °C. After incubation, 10 μl were mixed with the correspondent PS and plated on a sector of an agar plate which was incubated for 18 h at 30 °C.

RESULTS

Lysogeny of S. saprophyticus

With the methodology used for the induction and cross-spotting of *S. saprophyticus*, 26 of 100 strains were found to be lysogenic (Table 1). The corresponding 26 phages were propagated and stored as described. The stored phages were stable for a period of 4–6 months after which a significant fall in titre occurred.

S. saprophyticus *phage groups*

Phage typing at RTD × 100 of the 100 *S. saprophyticus* strains showed that most of the reactions in typable strains were + + or CL with at least one of the phages. It was apparent that with 12 phages three associations could be frequently seen and they were named lytic groups A, B and C, which included 75% of all typable strains. Group A was composed by phages ϕS61 and ϕS72, Group B by phages ϕS3, ϕS20, ϕS50, ϕS83, ϕS85, ϕS97, and finally Group C by phages ϕS23. ϕ41, ϕS63 and ϕS68. Table 2 shows the individual phages, number of strains and corresponding percentage referent to each lytic group. The lytic spectrum of most phages was different within each lytic group with at least one of the phages.

Serological relationships among phages

It was not possible to establish any relationship between the provisional serological phage groups and lytic groups. Phages ϕS3, ϕS50, ϕS72 and ϕS85 proved to be serological identical. Each of the four antisera neutralized the four phages at RTD in the described conditions. The four phages were not neutralized by serum anti-ϕS68 and lysed their own PS. Reciprocally neither of the four antisera neutralized ϕS68. Apparently, as occurs with *S. aureus* phages, there is no corrspondence between any of the proposed three lytic groups and any two provisional serological groups suggested above.

Evaluation of S. saprophyticus *phage set*

Typability at RTD × 100 was 45·1%. Reproducibility of lytic reactions of phages was evaluated by repeat testing of 12 selected strains showing different lytic reactions. Identical reactions with each phage were obtained from 94·2% to 100% in 120 tests. Discrimination analysis using 100 random pairs of strains showed that 88 pairs could be differentiated by 2 strong lytic reactions, 9 pairs by 1 strong reaction and 3 pairs were indistinguishable.

Table 1. *Lysogenic strains, phage designation, cross-spotting and selected propagating strains from a group of 100* S. saprophyticus *strains*

Lysogenic strains	Phage designation	Cross-spotting	Propagating strains
S3	ϕS3	S19 CL, S27 CL, S46 + +, S47 + +, S52 CL, S74 CL, S92 CL, S97 CL	PSS19
S14	ϕS14	S18 ±	PSS18
S18	ϕS18	S34 + +	PSS34
S20	ϕS20	S19 CL, S52 CL, S68 + +, S74 CL	PSS19
S22	ϕS22	S65 + +, S99 + +	PSS65
S23	ϕS23	S16 CL, S89 CL	PSS89
S29	ϕS29	S26 +, S89 + +	PSS26
S38	ϕS38	S44 ±	PSS44
S40	ϕS40	S16 + +, S42 CL, S80 +, S96 +	PSS42
S41	ϕS41	S48 + +, S89 CL S100 + +	PSS48
S44	ϕS44	S34 CL	PSS34
S46	ϕS46	S26 ±, S89 + +	PSS26
S48	ϕS48	S25 +, S71 CL, S80 CL, S89 + +, S96 CL, S100 + +	PSS25
S50	ϕS50	S74 ±	PSS74
S60	ϕS60	S89 CL	PSS89
S61	ϕS61	S11 CL, S16 CL, S37 CL, S43 + +, S51 CL, S55 CL, S65 CL, S72 CL, S83 + +, S89 CL, S91 CL, S92 +, S95 CL	PSS11
S63	ϕS63	S89 CL, S96 CL	PSS89
S68	ϕS68	S17 +	PSS17
S69	ϕS69	S34 CL	PSS34
S72	ϕS72	S37 CL, S65 CL, S95 CL	PSS95
S83	ϕS83	S19 CL	PSS19
S85	ϕS85	S19 CL, S27 + +, S29 + +, S46 +, S47 + +, S52 + +	PSS19
S88	ϕS88	S34 + +	PSS34
S90	ϕS90	S44 +	PSS44
S93	ϕS93	S44 + +	PSS44
S97	ϕS97	S19 CL, S52 CL, S68 + +, S74 CL, S80 + +	PSS52

Table 2. *Three* S. saprophyticus *phage groups observed with 60 typed strains of a group of 100 selected strains*

Phage group	Individual phages	No. of typable strains (and % of phage group among them)
A	ϕS61/ϕS72	15 (33·3 %)
B	ϕS3/ϕS20/ϕS50/ϕS83/ϕS85/ϕS97	16 (35·5 %)
C	ϕS23/ϕS41/ϕS63/ϕS68	5 (11·1 %)
Mixed		9 (20 %)
Non-grouped		15*

* Only 25 % of typable strains did not belong to any phage group A, B, or C.

Phage typing of other Staphylococcus *species using* S. saprophyticus *phage set*

Table 3 shows the results of phage typing 502 staphylococcus strains of different species using the set of 26 phages. As shown, 105 of 207 strains of *S. saprophyticus* isolated from UTI (50·7 %) were typable in comparison with 29 of 90 (32·3 %)

Table 3. *Phage typing of 502* staphylococcus *strains using* S. saprophyticus
phage set

Staphylococcal species	No. of tested strains	No. of typable strains (and percentage)
S. saprophyticus		
Isolated from urine	207	105 (50·5 %)
Isolated from skin	90	29 (32·3 %)
S. cohnii	47	6 (12·7 %)
S. xylosus	10	1
S. epidermidis	67	0
S. aureus	81	2*

* Strains *W57* (old multi-sensitive) and *80 CR5Rif* (restriction enzymes deficient mutant). both lysed only by phage ϕS48 (See Discussion).

strains isolated from skin and mucous membranes. Twelve percent of *S. cohnii* and one strain of *S. xylosus*, were typable. *S. epidermidis* and *S. aureus* were untypable with two exceptions.

DISCUSSION

The phages isolated in this study proved to be fairly specific for *S. saprophyticus*. The phage set typed 134 (45·1 %) of 297 *S. saprophyticus* strains. However, its specificity was not absolute since 6 of 47 *S. cohnii* strains and 1 of 10 *S. xylosus* strains were also lysed. With two exceptions neither coagulase positive nor *S. epidermidis* strains were lysed. Only phage ϕS48 lysed two *S. aureus* strains with special characteristics, strain W57 (frequently used as an indicator strain because of its high susceptibility to phages) and strain 80CR5Rif (a restriction-deficient strain). These were used to confirm lysis by *S. saprophyticus* phages; this also occurs with most *S. aureus* phages and some *S. epidermidis* phages.

The results obtained in the evaluation of this *S. saprophyticus* phage set (typability, reproducibility and discrimination) are similar to those obtained with phages for typing *S. epidermidis* [8–10, 14, 19]. However, the results of discriminatory analysis must be considered with some caution because the strains from which the random pairs were selected did not have known epidemiologic relationships. With continued *S. saprophyticus* typing some phages may be discarded.

Melo Cristino and Torres Pereira [20] studied the occurrence of *S. saprophyticus* in the indigenous flora of different areas of the skin and mucous membranes of 451 healthy individuals and patients. *Staphylococcus saprophyticus* was a member of the indigenous flora in the areas studied, but in all cases numbers were very low compared not only with the total number of staphylococci but also with other novobiocin resistant species. The phage typing technique described in this paper may be useful as an ecological tool and to trace the origin of urinary tract strains from the skin or mucous membranes.

Finally since Melo Cristino, Torres Pereira and Andrade [21] described a diversity of plasmids in *S. saprophyticus* isolated from UTI, with 82 % of the strains harbouring plasmids, some of them with complex plasmid profiles, we anticipate that phage typing in association with plasmid profiling may be

complementary methods with strong discriminatory power for differentiating among *S. saprophyticus* strains.

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