Factors affecting conjugative transfer of plasmid pWG613, determining gentamicin resistance, in *Staphylococcus aureus*

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Summary. Factors that are known to influence plasmid transfer in bacterial populations were studied for the conjugative plasmid pWG613, which determined gentamicin resistance in *Staphylococcus aureus*. The transfer frequency was largely unaffected over a wide range of temperature (18–42°C); pH also had little effect on the transfer frequency in the range 5.0–8.5. High cell density and log phase cultures were required for optimal plasmid transfer, as were donor: recipient ratios of 0.003–3.3.

Introduction

Soon after the introduction of antibiotics into clinical practice, it became clear that *Staphylococcus aureus* could develop resistance to many agents.^{1–3} Antibiotic resistance in this organism is often plasmid-encoded and may be transferred in the laboratory by transformation, transduction or conjugation.^{4–6} Plasmid transfer by phage-mediated conjugation (which requires the presence of phage in either donor or recipient without death of the former or lysis of the latter) occurs at a high frequency (10^{-1} per recipient) in mixed cultures and might also take place under natural conditions.^{7, 8} In the last few years conjugative transfer of plasmids determining aminoglycoside resistance has been demonstrated between *S. epidermidis* and *S. aureus*.^{6, 9–12} This finding could be of clinical significance and may explain the increased incidence of gentamicin resistance in staphylococci.^{13–17}

Various factors influence conjugative plasmid transfer amongst bacteria. These include temperature, pH,

cell density and the donor:recipient cell ratio.^{18–24} Very few reports have considered the effects of these factors in staphylococci. The aim of this work was to study their influence on the conjugative transfer of an aminoglycoside resistance plasmid to evaluate the potential for self-transmissible plasmid exchange among staphylococci.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in the table. All were maintained on Nutrient Agar (Oxoid CM3) containing appropriate selective agents. Liquid cultures were grown in 10-ml volumes of Nutrient Broth (NB; Oxoid CM1). Nutrient Agar (NA; Oxoid), Brain Heart Infusion Agar (BHIA; Oxoid CM375), Diagnostic Sensitivity Test Agar (DSTA; Oxoid CM261) and Blood Agar (BA) were

Table. Source and properties of *S. aureus* strains and mutants

Strain or mutant no.	Properties	Resistance phenotype	Reference
WG2726	Clinical isolate 661 carries pWG613 (41.6 Kb)	Gm ^r , Km ^r , Nm ^r , Cet ^r , EB ^r	6, 25
RN450	Sensitive strain	None	26
SAU1	Rif ^r , Fd ^r mutant of RN450	Rif ^r , Fd ^r	This paper
SAU2	Rif ^r , Sm ^r mutant of RN450	Rif ^r , Sm ^r	This paper
SAU3	SAU1 (pWG613)	Gm ^r , Km ^r , Nm ^r , Cet ^r , EB ^r	This paper

Cet, cetrимide; EB, ethidium bromide; Fd, fucidic acid; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Rif, rifampicin; Sm, streptomycin.

used as culture media. Blood Agar comprised Columbia Blood Agar (Oxoid) plus horse blood 5%.

Mating procedure

The *S. aureus* donor SAU3 and recipient SAU2 strains were grown separately overnight in NB, then diluted 10-fold into pre-warmed fresh medium and incubated for 4 h at 37°C. Equal (0.25-ml) volumes of these cultures were mixed in 2.5 ml of fresh NB and filtered through cellulose acetate membrane filters (0.45 µm pore size, 25 mm diameter; Oxoid). The filters were incubated, face up, on the surface of NA for 15 h at 37°C. The cells on the filters were then resuspended by vortex mixing for 2 min in 1 ml of NB. Serial dilutions of the washings were plated on the appropriate selective media and incubated at 37°C for 48 h. These media were NA containing gentamicin 5 mg/L, streptomycin 60 mg/L or both these antibiotics. In some experiments cetrимide (5 mg/L) replaced gentamicin. Separate cultures of the donor and recipient strains were treated identically and in parallel as controls.

Colonies growing on the NA containing both antibiotics were classed as presumptive transconjugants and were counted after incubation for 48 h. The transfer frequency was calculated relative to the counts of recipient cells present at the start of mating. Transconjugants were purified on media containing gentamicin plus streptomycin and replicated on to media containing other antimicrobial agents to test for co-acquisition of unselected markers.

Factors affecting the transfer frequency

The standard mating procedure was used. The effects on the transfer frequency of the incubation temperature prior to mating, and during mating; pH; mating duration; cell density; the donor:recipient ratio and different media were investigated.

Statistical analyses

All transfer frequencies were the means of at least three determinations. Minimum significant ranges (MSR) were calculated by Tukey's honestly significant difference method.²⁷ All differences were considered significant if $p < 0.05$.

Results

Effects of different media on plasmid transfer

Matings were performed on NA, BHIA, DSTA and BA. There was no significant difference in the transfer frequencies on any of the first three media (1.6×10^{-1} to 7.3×10^{-2} per recipient), but the frequency on BA was significantly ($p < 0.05$) lower (5.4×10^{-3} per recipient).

Effects of the mating period on plasmid transfer

Transfer of the plasmid was first detected after 1 h and reached a maximum frequency after 6 h (fig. 1). Increasing the mating period to 24 h gave no further significant increase in the transfer frequency.

Effects of temperature on plasmid transfer

The influence of temperature on the transfer of pWG613 was investigated in two ways. Firstly the effect of the growth temperature before mating was examined. Individual donor and recipient cultures were grown separately for 4 h at various temperatures from 12 to 42°C. Donor and recipient cultures grown at the same temperature were then mixed and

Fig. 1. The effect of the duration of mating period on transfer of plasmid pWG613. Transfer frequencies per recipient cell (□—□) and per donor cell (■—■) present at the start of mating are shown. The bar represents the minimum significant range (MSR).

Fig. 2. The effect of growth temperature prior to mating on transfer of plasmid pWG613. The results are expressed per recipient cell present at the start of mating (□—□). The numbers of recipient cells initially present (■—■) and transconjugants obtained (△—△) are also shown. The bar represents the minimum significant range (MSR).

harvested by filtration. The filters were incubated at 37°C for 15 h. There was no significant temperature-dependent difference in the transfer frequency. Transfer frequencies were between 1.5×10^{-1} and 4.0×10^{-2} per recipient (fig. 2).

In other experiments, the donor and recipient cells were grown separately at 37°C for 4 h, mixed and harvested on membrane filters. Individual filters were then incubated at temperatures between 10 and 42°C. There was no difference in the maximal transfer frequency observed between 25 and 42°C (fig. 3). Transfer of the plasmid was significantly reduced, but remained detectable at 18°C but not at 10°C.

Effect of pH on plasmid transfer

Transfer of pWG613 was not affected by pH values between 5.0 and 8.5 at 25 or 37°C. Fig. 4 shows the

Fig. 3. The effect of mating temperature on transfer of plasmid pWG613. Results are expressed per recipient cell present at the start of mating (□—□). The transconjugant (△—△) and total recipient counts (■—■) at the end of mating are also shown. The bar represents the minimum significant range (MSR).

Fig. 4. The effect of pH on transfer frequency of plasmid pWG613. Results are expressed per recipient cell (□—□) present at the start of mating. Transconjugant (△—△) and recipient counts (■—■) at the end of mating are also shown. The bar represents the minimum significant range (MSR).

transfer frequencies recorded at 37°C; similar data were obtained at 25°C.

Effect of culture density on plasmid transfer

The influence of the culture density on the transfer frequency was calculated. Maximum frequencies were obtained when the initial total (donor plus recipient) cell density was 1.8×10^7 cfu/ml. The lowest initial density at which transfer was detected was 1.0×10^4 cfu/ml. At initial densities of 1.8×10^2 cfu/ml no transfers were detected although the cell density had increased to 2.3×10^9 cfu/ml after 15 h (fig. 5). At high initial cell densities (1.0×10^9 cfu/ml) the transfer frequency was reduced (fig. 5). Cell densities at the end of the 15-h mating period were (2.0×10^9) – (2.0×10^{10}) cfu/ml and were not significantly dependent ($p > 0.05$) on the cell density at the start of mating.

Effect of the donor: recipient cell ratio on plasmid transfer

Transfer of the gentamicin resistance plasmid occurred at all the donor: recipient ratios used. Fig. 6 clearly shows that the maximum transfer frequencies were obtained over a wide range of initial donor: recipient cell ratios.

Effect of selective markers on plasmid transfer

Transcripts were selected on agar containing gentamicin or cetrимide together with streptomycin. The transfer frequency did not vary significantly according to the selective agent used ($p < 0.05$).

Fig. 5. The effect of cell density on the transfer frequency of plasmid pWG613. Results are expressed per recipient cell present at the start of mating (□—□). Transconjugant (△—△) and recipient counts (■—■) at the end of mating are also shown. The bar represents the minimum significant range (MSR).

Fig. 6. The effect of the donor: recipient ratio on transfer frequency of plasmid pWG613. The transfer frequency is expressed per recipient (■—■) and per donor (□—□) present at the start of mating. The total transconjugant count (△—△) is also shown. The bar represents the minimum significant range (MSR).

Transcipients invariably acquired resistance to both gentamicin and centrimide.

Discussion

Factors that might modify the transfer efficiency of the conjugative *S. aureus* plasmid pWG613 were examined. Similar transfer frequencies were obtained on NA, BHA, or DSTA but a decreased transfer frequency was noted on BA, probably caused by serum components which affect staphylococcal aggregation. This effect could be significant *in vivo*.

Transfer of plasmid pWG613 occurred within 60 min on filter membranes (fig. 2) and was maximal within 6 h. The relationship between the total number of transconjugants and time (data not shown) indicated that the transfer frequency peaked at the eighth generation. The temperature of incubation before mating was not important in determining the frequency of plasmid transfer. Similarly, the mating temperature was not important in determining the rate of plasmid transfer over the range 25–42°C. The transfer frequency was reduced at low temperature (18°C) but remained high in absolute terms (1.5×10^{-5} per recipient). Transfer between staphylococcal populations could therefore take place at room temperature. The spread of plasmids determining aminoglycoside resistance in some hospitals might be explained by their ability to transfer amongst strains both in patients and in the environment.

The influence of pH was investigated at 25 and 37°C. It has been reported elsewhere that pH and temperature can co-determine the plasmid transfer frequency.²³ However, transfer of pWG613 remained similarly efficient at pH values from 5.0 to 8.5. Curtiss²⁸ has also reported that drug resistance

plasmids in *Escherichia coli* were transferred optimally over the same pH range.

In conjugation experiments, initial cell densities of 10^4 – 10^8 cfu/ml were required to achieve high rates of plasmid transfer. At a higher or lower cell density the transfer frequency was depressed. The optimum transfer of plasmid pWG613 occurred at donor:recipient cell ratios between 0.003 and 0.3 (fig. 6).

This flexibility may help to account for the rapid "evolution" of gentamicin-resistant *S. aureus* strains through plasmid transfer in hospitals. A better understanding of this transfer process will help determine strategies for reducing the spread of resistance.

We gratefully acknowledge support from the Department of Biology, King Abdulaziz University and the Ministry of Higher Education, Saudi Arabia.

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