Excision of a conjugative plasmid from the staphylococcal chromosome

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Summary. *Staphylococcus aureus* isolate WBG1003 resistant to benzyl penicillin, cadmium, arsenate and streptomycin harbours two plasmids of 38.8 (pWBG621) and 4.4 (pWBG625) kb. In conjugation experiments two types of streptomycin-resistant transconjugants were obtained; one carried a 4.4-kb plasmid and the other, a 34.5-kb and a 4.4-kb plasmid. The 34.5-kb plasmid (pWBG620) has been found to be conjugative and able to mobilise non-conjugative plasmids. It has no detectable resistance phenotype and has not been detected in WBG1003 nor in the recipient used in the conjugation experiments. Restriction endonuclease analysis and DNA-DNA hybridisation have revealed that pWBG620 is unrelated to pWBG621 present in strain WBG1003. The data presented indicate that pWBG620 is in the chromosome of strain WBG1003 and that it excises during conjugation.

Introduction

Multi-resistant *Staphylococcus aureus* strains have continued to be a major cause of serious infections in man, both in hospitals and in the community.¹ Although resistance can be transferred between strains in the laboratory by transduction, mixed-culture transfer and conjugation, it has been suggested that conjugation is the most likely method of genetic exchange *in vivo*² and has been demonstrated between staphylococci on the human skin.³

Three types of conjugative plasmids have now been described in *S. aureus*: those encoding gentamicin, kanamycin and neomycin resistance and sometimes penicillinase production;^{4–8} those producing diffusible pigment (DiP) and encoding resistance to macrolides, lincosamides and streptogramin B and sometimes to kanamycin, neomycin and streptomycin;^{9,10} and those without any resistance phenotype.^{11,12} These plasmids have been demonstrated in the host cells and, with the exception of the DiP plasmids, mobilise non-conjugative plasmids. It has also been reported that resistance to gentamicin¹³ and mupirocin¹⁴ can be transferred conjugatively in the absence of detectable conjugative plasmids.

In this paper we report the conjugative transfer

of streptomycin resistance in the absence of a detectable conjugative plasmid in the donor or in the recipient prior to conjugation. Evidence is presented that the donor contains a conjugative plasmid in its chromosome which excises during conjugation and mobilises a non-conjugative, streptomycin-resistance plasmid.

Materials and methods

Bacteria and plasmids

Bacterial strains and plasmids used are listed in table I.

Media

Brain Heart Infusion Broth (BHIB), Brain Heart Infusion Agar (BHIA), Trypticase Soy Broth (TSB) and Mueller Hinton Agar (MHA) were purchased from Gibco Diagnostics (Madison, WI, USA).

Susceptibility testing

Disk diffusion. This was performed on MHA as described previously.^{15–17}

Replica plating. The method of Annear and Grubb¹⁸ was used to replicate to MHA plates containing cadmium (Cd; 10^{-2} M), streptomycin (Sm; 30 gm/L) or benzylpenicillin (Pc; 2000 U/L). Penicillinase production was determined with nitrocefin.¹⁹

Table I. *S. aureus* strains and plasmids

Strain no.	Description	Reference
WBG248	Plasmid free	(15)
WBG541	Fa ^r Rf ^r mutant of WBG248	(15)
WBG742	Nb ^r mutant of WBG248	(9)
WBG1876	WBG541 lysogenised with phage J	(16)
WBG4483	Plasmid mol. wt markers	(20)
WBG4515	Nb ^r Sm ^r mutant of WBG248	(11)
WBG1003	Clinical isolate; Pc ^r , Cd ^r , Asa ^r ; Sm ^r ; harbours plasmids pWBG621 and pWBG625	
pWBG621	Codes resistance for Pc, Cd, Asa; 38.8 kb	
pWBG625	Codes resistance to Sm; 4.4 kb	
WBG4839	WBG1003 cured of pWBG625	
WBG4840	WBG1003 cured of pWBG621	
WBG4841	Sm ^r transconjugant of WBG1003/541; harbours pWBG625	
WBG4842	Sm ^r transconjugant of WBG1003/541; harbours pWBG625 and pWBG620	
pWBG620	Plasmid (34.5 kb), detected after conjugation	
WBG4843	WBG4842 cured of pWBG625	
WBG4843-1	Transconjugant WBG4840/541; harbours plasmid pWBG620-1 (34.5 kb)	
WBG4844	WBG4843 carrying pWBG3 (4.4 kb, Tc ^r)	
WBG4845	WBG4843 carrying pE194 (3.5 kb, Em ^r)	
WBG4881	WBG541 carrying pWBG3	(11)
WBG4882	WBG541 carrying pE194	(11)
WBG4858	WBG541 carrying pWBG637 (34.5 kb)	(11)

Asa, arsenate; Cd, cadmium; Em, erythromycin; Fa, fusidic acid; Nb, novobiocin; Pc, benzylpenicillin; Rf, rifampicin; Sm, streptomycin; Tc, tetracycline.

Bacteriophage typing

Phage typing was performed by Dr D. Perret at the State Health Laboratory Services of Western Australia, Perth.

Loss of resistance at 43.5°C

Strains were grown either on BHIA or in BHIB at 43.5°C for 24 h, plated on to BHIA plates and incubated at 37°C overnight. Single colonies were then screened for loss of resistance to antimicrobial agents by the replica plating method. These results were confirmed by the disk-diffusion method.

Transfer of plasmids

Transduction was performed as described previously¹⁵ with phage 85. Mixed culture transfer (MCT) and conjugation experiments were performed as described previously.^{16, 20} Briefly, in MCT, 0.1 ml of overnight cultures of donor and recipient were added to 5.0 ml of TSB containing 0.04 M CaCl₂. The mixture was incubated overnight at 37°C with gentle shaking and then the cells were pelleted by centrifugation. The supernate was discarded and the deposit was spread on BHIA plates containing appropriate selective agents. Selection for resistance was made on plates of either Cd (10⁻² M) or

Sm (10 gm/L). The recipient for MCT was strain WBG1876 which was lysogenised with phage J.¹⁶

Conjugation studies were performed by the polyethylene glycol (PEG) method²⁰ with strain WBG541, a non-lysogenic recipient. Equal volumes (2 ml) of an overnight culture of each of the donor and recipient were mixed in a tube and pelleted by centrifugation. The supernate was discarded and the deposit was resuspended in 0.5 ml of TSB to which was added 5.0 ml of PEG 40% w/v. The tube was then incubated at 37°C overnight with gentle shaking. The cells were pelleted by centrifugation and resuspended in 1.0 ml of TSB. From this, serial 10-fold dilutions were made and 0.1 ml of each dilution was spread on selective media. In both MCT and conjugation experiments, controls consisting of donor and recipients alone were incorporated for each experiment. Transfer frequencies were given as the number of transconjugants in relation to the number of donor cells at the beginning of the experiment. Transfer was considered to have occurred when growth was obtained on selection plates from donor-recipient mixtures but not from selection plates inoculated with the donor and recipient controls.

Plasmid isolation

Plasmids were isolated by the cetyltrimethylammonium bromide (CTAB) method.²¹ DNA obtained by this method was usually suitable for restriction endonuclease

analysis. When further purification was necessary, large amounts of plasmid DNA were isolated by the CTAB method and purified by caesium chloride-ethidium bromide density centrifugation.¹² Horizontal agarose-gel electrophoresis and restriction-enzyme analysis have been described previously.¹² Vertical gel electrophoresis was performed in TBE buffer (445 mM Tris base, pH 8.0, 445 mM boric acid, 10 mM EDTA).²²

DNA-DNA hybridisation

This was performed as described previously¹² except that Genescreen Plus (Dupont, Boston, USA) filter membranes were used instead of nitrocellulose.

Results

Properties of strain WBG1003

S. aureus strain WBG1003 was found to be resistant to benzyl penicillin (Pc), cadmium (Cd), arsenate (Asa) and streptomycin (Sm) and sensitive to erythromycin (Em), lincomycin, tetracycline (Tc), gentamicin, kanamycin, neomycin, chloramphenicol, rifampicin (Rf), fusidic acid (Fa), mercury, phenylmercuric acetate and to the nucleic-acid binding (NAB) compounds such as propamidine isethionate, cetyltrimethylammonium bromide and ethidium bromide.¹⁷ It was untypable with phages of the International Phage Typing Set and contained plasmids of 38.8 (pWBG621) and 4.4 (pWBG625) kb (fig. 1).

Location of resistance determinants

Loss of the resistance determinants after growth at 43.5°C indicated that all the determinants were plasmid-borne. Resistance to Pc, Cd and Asa were lost conjointly whereas Sm resistance (Sm^r) was lost separately. Loss of Sm^r corresponded to the loss of the 4.4-kb plasmid (pWBG625) and loss of Pc^r, Cd^r, Asa^r to the loss of pWBG621 (38.8 kb, fig. 1).

Transfer of resistance determinants

Plasmid transfer in MCT experiments occurs when either the donor or recipient is lysogenised with a suitable bacteriophage.²³ Since WBG1003 was not lysed by phages of the International Typing Set, MCT was attempted with strain WBG1876 (lysogenised with phage J, 16) as recipient. Strain WBG1876 has been used successfully as recipient in previous MCT experiments with non-typable isolates of *S. aureus*.¹⁶ None of the resistance determinants of strain WBG1003 was transferred.

When strain WBG1003 was tested in conjugation

Fig. 1. Plasmids of strain WBG1003. Lane 1: (a) covalently-closed circular pWBG621, 38.8 kb; (b) covalently-closed circular pWBG625, 4.4 kb. 2: strain WBG1003 cured of pWBG621 (WBG4840); 3: strain WBG1003 cured of pWBG625 (WBG4839).

experiments with strain WBG541 and selection was made for Sm^r and Cd^r, transconjugants were obtained on media with Sm but not Cd selection (table II). None of 227 Sm^r transconjugants screened co-transferred resistance to Pc, Cd and Asa. Gel electrophoresis of the Sm^r transconjugants revealed two plasmid profiles. About one-third harboured a single 4.4-kb plasmid whereas the remainder contained a 34.5-kb plasmid (pWBG620) and a 4.4-kb plasmid (fig. 2). These results indicate that the 4.4-kb plasmid in the transconjugants was the Sm^r plasmid, pWBG625, from strain WBG1003. A transconjugant containing only pWBG625 was designated strain WBG4841 and one containing both pWBG620 and pWBG625, strain WBG4842 (table I).

Analysis of strain WBG4842

Strain WBG4842 harboured two plasmids but had only acquired resistance to Sm. Although

Table II. Transfer of resistance and plasmids by conjugation

Donor strain	Recipient strain	Selective agents	Resistance and plasmids (kb) transferred	Frequency
WBG1003	WBG541	FaRfCd	None	$<1.0 \times 10^{-9}$
WBG1003	WBG541	FaRfSm	Sm, 4.4	1.6×10^{-6}
WBG1003	WBG541	FaRfSm	Sm, 4.4; 34.5	1.6×10^{-6}
WBG4840	WBG541	FaRfSm	Sm, 4.4	1.2×10^{-6}
WBG4840	WBG541	FaRfSm	Sm, 4.4; 34.5	1.2×10^{-6}
WBG4841	WBG742	NbSm	None	$<1.0 \times 10^{-9}$
WBG4842	WBG742	NbSm	Sm, 4.4	1.4×10^{-6}
WBG4842	WBG742	NbSm	Sm, 4.4; 34.5	1.4×10^{-6}
WBG4844	WBG4515	NbSmTc	Tc, 4.4	1.0×10^{-6}
WBG4844	WBG4515	NbSmTc	Tc, 4.4, 34.5	1.0×10^{-6}
WBG4845	WBG4515	NbSmEm	Em, 3.5	1.2×10^{-7}
WBG4845	WBG4515	NbSmEm	Em, 3.5; 34.5	1.2×10^{-7}
WBG4881	WBG4515	NbSmTc	None	$<1.0 \times 10^{-9}$
WBG4882	WBG4515	NbSmEm	None	$<1.0 \times 10^{-9}$

Cd, cadmium; Em, erythromycin; Fa, fusidic acid; Rf, rifampicin; Nb, novobiocin; Sm, streptomycin; Tc, tetracycline.

Fig. 2. Strain WBG1003 and its transconjugants. Lane 1, strain WBG1003; 2, strain WBG4842 (pWBG620 and pWBG625); 3, strain WBG4843 (pWBG620); 4, strain WBG4845 or WBG4845/WBG4515 transconjugants containing pWBG620 and pE194; 5, strain WBG4844 (pWBG620 and pWBG3); 6, WBG4844/WBG4515 transconjugant, carrying both pWBG3 and pWBG620; 7, WBG4844/WBG4515 transconjugant carrying only pWBG3; 8, same as lane 6; 9, strain WBG4483, plasmid mol.-wt markers (see fig. 5 for details) (pWBG615 is not visible in this gel).

pWBG625 encodes resistance to Sm, it is possible that the 34.5-kb plasmid pWBG620 also encodes Sm^r and that the two plasmids may be responsible for different levels of resistance. However, strains WBG1003, WBG4841 and WBG4842 all had the same MIC to Sm (75 µg/ml) when tested. It is also possible that pWBG620 does not encode or express Sm^r but is conjugative and was only mobilising pWBG625. To confirm this, strains WBG4842 and WBG4841 were tested separately in conjugation experiments with WBG742 and selection was made for Sm^r. Sm^r was transferred only from strain WBG4842, confirming that the 34.5-kb plasmid was conjugative and was mobilising pWBG625. When the WBG4842/WBG742 transconjugants were analysed for plasmid content they were found to be of two types, similar to those obtained when strain WBG1003 was the donor. One type carried a single 4.4-kb plasmid and the other both the 34.5-kb and 4.4-kb plasmids.

To study pWBG620 further, attempts were made to isolate it on its own. Strain WBG4842 was grown at 43.5°C for 48 h and single colonies were screened for loss of Sm^r. When Sm^s colonies were analysed for plasmid content, they were found to have lost both plasmids. Strain WBG4842 was then grown at 37°C for 48 h and single colonies were screened for loss of Sm^r. Two Sm^s colonies were obtained out of 168 examined. Plasmid analysis revealed that the Sm^s colonies had lost pWBG625 but retained pWBG620. One of these colonies was designated strain WBG4843 (table I; fig. 2).

Characterisation of *pWBG620*

Strain WBG4843, containing only *pWBG620*, was tested for resistance to antibiotics, heavy metals¹⁵ and NAB compounds.¹⁷ It was found to be resistant only to Rf and Fa, the two chromosomal resistance markers of strain WBG541.

The ability of *pWBG620* to mobilise plasmids other than *pWBG625* was studied by transducing *pWBG3* (Tc^r) and *pE194* (Em^r) to strain WBG4843 in separate experiments to produce strains WBG4844 and WBG4845 respectively. Strains WBG4844 and WBG4845 were conjugated with strain WBG4515 in separate experiments and selection was made for the phenotypes of the non-conjugative plasmids and the chromosomal resistances of strain WBG4515. In both cases the non-conjugative plasmids were transferred into strain WBG4515 (table II; fig. 2). When transconjugants were analysed for plasmid content, they were found to be of two types; one type carried only the non-conjugative plasmid and the other type carried both the conjugative and non-conjugative plasmids. Conjugation experiments were also performed with strain WBG4515 and donors carrying only the non-conjugative plasmids *pWBG3* (WBG4881) and *pE194* (WBG4882). No transfer was observed in either case, which confirms that both *pWBG3* and *pE194* would not transfer by conjugation in the absence of *pWBG620*.

Origin of *pWBG620*

As plasmid *pWBG620* cannot be detected in the parent strain WBG1003, and is only observed in transconjugants (fig. 2), this raises the question of its origin. Since strain WBG1003 harbours a 38.8-kb plasmid (*pWBG621*) encoding resistance to Pc, Cd and Asa, *pWBG620* is possibly related to it and results from the deletion of the resistance determinants during conjugation. If this is the case, then both plasmids, *pWBG620* and *pWBG621*, would have DNA sequences in common. However the plasmids did not have common fragments when digested with restriction enzymes *Bgl*II, *Cla*I, *Hpa*II (fig. 3) and *Eco*RI (fig. 4A) and no homology was detected when ³²P-labelled *pWBG620* was hybridised with *pWBG621* (fig. 4).

Any role for *pWBG621* was further discounted by conjugating strain WBG4840, a derivative of strain WBG1003 cured of *pWBG621*, with strain WBG541. Sm^r was transferred to strain WBG541 (table II; fig. 5) and the transconjugants were similar to those obtained when strain WBG1003 was used as donor. One of the 34.5-kb plasmids,

Fig. 3. Restriction endonuclease analysis of plasmids *pWBG621* and *pWBG620* with (A) *Bgl*II, (B) *Cla*I and (C) *Hpa*II. Lanes 1, *pWBG621*; 2, *pWBG620*.

pWBG620-1, from the WBG4840/WBG541 transconjugants was isolated and cut with *Eco*RI restriction enzyme. It gave the same sized fragments as *pWBG620* (not shown).

Since *pWBG620* appears not related to *pWBG621*, there would appear to be three possible explanations for its origin. Firstly, it could have been introduced by a contaminating strain such as WBG1024 carrying plasmid *pWBG637*.¹¹ Secondly, it could have been derived from a larger plasmid not detected in strain WBG1003, or thirdly it could have excised from the chromosome of the donor strain WBG1003.

To test if *pWBG620* was due to a contaminating strain, single colonies of WBG1003 were used in conjugation experiments using WBG541 as recipient. Two of seven single colony isolates transferred Sm^r. Analysis of the seven single-colony clones and

Fig. 4. Southern blot analysis of plasmids pWBG621 and pWBG620. (A) *Eco*RI analysis of plasmids pWBG621 (Lane 1) and pWBG620 (2). (B) Autoradiograph of gel in panel A after Southern transfer and hybridisation with 32 P-labelled pWBG620.

strain WBG1003 did not reveal any differences in plasmid content (not shown) which eliminates the possibility that some of the cells were carrying a plasmid of the pWBG637 type.

Plasmid pWBG620 was possibly derived from a large plasmid in strain WBG1003 which was not detected on horizontal gel electrophoresis. However, when the preparations were run on vertical gel electrophoresis, no additional plasmids were detected in strain WBG1003. These results are consistent with the hypothesis that pWBG620 is located on the chromosome of strain WBG1003 and that it excises during conjugation.

Comparison of plasmids pWBG620 and pWBG637

Plasmid pWBG620 appeared to be similar to pWBG637 which has been reported previously to be conjugative and without any resistance phenotype.¹¹ Both plasmids appeared to have the same

Fig. 5. WBG4840/WBG541 transconjugants. Lane 1, strain WBG4840 or representative of WBG4840/WBG541 transconjugants carrying only pWBG625; 2, representative of WBG4840/WBG541 transconjugants carrying both pWBG620-1 and pWBG625; 3, strain WBG4483, plasmid mol.-wt markers from top, open circular, OC, pWBG615, 40.3 kb; OC pWBG115, 22.5 kb; chromosomal DNA; covalently-closed, CCC, pWBG615; CCC pWBG115; OC pWBG3, 4.4 kb; OC pE194, 3.5 kb; CCC pWBG3; CCC pE194.

molecular weight and generated the same fragments when digested with *Eco*RI, *Hind*III, *Cla*I and *Hpa*II (not shown). Furthermore, pWBG620 hybridised with a 32 P-labelled pWBG637 probe in a Southern hybridisation experiment¹² which indicates that the two plasmids are identical or very similar.

Discussion

Only two plasmids have been detected in strain WBG1003—one of 4.4-kb encoding *Sm*^r (pWBG625) and one of 38.8-kb encoding *Pc*^r, *Cd*^r, *Asa*^r (pWBG621). Since strain WBG1003 is untypable with phages of the International Typing Set, it was not possible to transduce the plasmids. Attempts to transfer the plasmids in MCT were also

unsuccessful. However, pWBG625 was transferred during conjugation. Its transfer was due to a 34.5-kb plasmid, pWBG620, which was not detectable in strain WBG1003 and had no homology with pWBG621, the 38.8-kb plasmid found in strain WBG1003. When conjugation was performed with strain WBG4840, the parent strain WBG1003 lacking pWBG621, pWBG625 was mobilised by a plasmid indistinguishable from pWBG620. There is no evidence that a third plasmid exists extra-chromosomally in strain WBG1003. Plasmid pWBG620 was not detected on horizontal or vertical gels. Vertical gel electrophoresis has been used to separate large mol.-wt plasmids (> 100 kb) in gram-negative bacilli.^{24, 25} To our knowledge, no plasmids of this size have been reported in *S. aureus*. The implication, from these results, is that pWBG620 is integrated in the chromosome of strain WBG1003, excises during conjugation and mobilises pWBG625.

Plasmid pWBG620 has no detectable resistance phenotype and appears to be indistinguishable from pWBG637, a conjugative plasmid described in another isolate.¹¹ This similarity was confirmed by restriction enzyme analysis and DNA-DNA hybridisation.¹² However, there is no evidence that pWBG 637 is able to integrate into the chromosome of its host strain WBG1024. Also, neither pWBG637 nor pWBG620 appears to be able to integrate into the chromosome of transconjugants. In every case examined, plasmids could be mobilised only from transconjugants if either pWBG637 or pWBG620 was detected. Consequently, it would appear that strain WBG1003 must have some property, such as a specific integration site, which enables pWBG620 to integrate into the chromosome. Once pWBG620 has excised or mobilises another plasmid, it appears to be unable to integrate into the chromosome of the transconjugants. This may be how pWBG637 arose initially.

The nature of pWBG620 within the chromosome of strain WBG1003 has not yet been determined. Either it exists as an integrated plasmid and excises precisely during conjugation, or it is formed as a result of excisive recombination between insertion sequences (IS) in the chromosome. Different IS elements have been shown to be involved in the excision of Type-II F-prime plasmids in *Escherichia*

coli^{26, 27} and cryptic plasmids in *Pseudomonas syringae* pv *phaseolicola*.²⁸ DNA-DNA hybridisation analysis of the chromosome of strain WBG1003 with various fragments of pWBG620 as probes will provide physical evidence of the integration of pWBG620. Inability of five of the seven single colonies examined to transfer Sm^r could be due to the absence of pWBG620 in their chromosomes or the integrated plasmids are excision defective. The probes of pWBG620 could also be used to clarify the situation. In the meantime the results presented provide genetic evidence that pWBG620 does excise from the chromosome of strain WBG1003 during conjugation.

Although cryptic conjugative plasmids capable of excising from the chromosome have been reported in *Streptomyces* spp.,²⁹⁻³² *Haemophilus influenzae*,³³ *Lactobacillus acidophilus*³⁴ and the plant pathogen *P. syringae*,^{28, 35} this is the first time a conjugative plasmid has been reported to excise from the chromosome in staphylococci.

These results may explain reports of other resistance determinants being transferred during conjugation in the apparent absence of a conjugative plasmid. Recent examples are the transfer of gentamicin¹³ and mupirocin¹⁴ resistances in the absence of detectable conjugative plasmids either in the donor or in the transconjugants. This does not however exclude the possibility that conjugative transposons may also be involved.

Until recently the only conjugative plasmids reported in the staphylococci are those conferring resistance to gentamicin and kanamycin⁴⁻⁸ and the DiP plasmids.^{9, 10} The discovery of a new class of conjugative plasmids which do not have any resistance phenotype and which are able to mobilise non-conjugative plasmids,^{11, 12} further extends the scope of resistance transfer in the staphylococci. The finding that this class of plasmid probably can excise from the chromosome, at least of some staphylococci, raises the possibility that they may be able to transfer chromosomal determinants.

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