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Staphylococcus aureus Bacteriophages Mediating the Simultaneous Lysogenic Conversion of β-Lysin, Staphylokinase and Enterotoxin A: Molecular Mechanism of Triple Conversion

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A new group of serotype F bacteriophages of *Staphylococcus aureus* has been found which mediates the simultaneous triple-lysogenic conversion of enterotoxin A, staphylokinase and β-lysin. The phages were recovered from methicillin-resistant strains of *S. aureus* isolated in Irish hospitals between 1971 and 1988 and from strain PS42-D, which has been used as the propagating strain for the *S. aureus* typing phage 42D since before 1965. The molecular mechanism of triple conversion mediated by three of these phages was determined by molecular cloning, restriction endonuclease site mapping and hybridization analysis, and compared with the mechanism of β-lysin and staphylokinase conversion mediated by the serotype F, double-converting phage φ13. The genetic determinants mediating expression of enterotoxin A (*entA*) and staphylokinase (*sak*) were cloned from the DNA of the triple-converting phage and expression of the cloned determinants detected in *Escherichia coli* and *S. aureus*. The *entA* and *sak* determinants were closely linked in the phage DNA adjacent to the phage attachment site (*attP*) in each case and furthermore, the *sak* determinant of phage φ13 was also located near its *attP*. The restriction maps of the *entA*-, *sak*- and *attP*-containing DNA regions of the three triple-converting phages were very similar to each other and to the corresponding *sak*- and *attP*-containing DNA region of phage φ13. Hybridization analysis using a cloned β-lysin determinant (*hlb*) and cloned *attP*-containing DNA fragments as probes demonstrated that β-lysin conversion mediated by the triple-converting phages and phage φ13 was caused by insertional inactivation of the chromosomally encoded *hlb* determinant by orientation-specific integration of phage DNA following lysogenization.

INTRODUCTION

Carriage of lysogenic prophages by *Staphylococcus aureus* strains is common. Lysogeny can affect the expression of several extracellular toxins and enzymes produced by these organisms, some of which may be important virulence factors (Parker, 1983). Expression of β-lysin and lipase can be lost following lysogenization (negative phage conversion) (Winkler *et al.*, 1965; Lee & Iandolo, 1985; Coleman *et al.*, 1986), whereas the capacity to express staphylokinase and enterotoxin A can be acquired after lysogenization (positive phage conversion) (Winkler *et al.*, 1965; Casman, 1965). Concomitant conversion of multiple phenotypic properties of some *S. aureus* strains following lysogenization has also been described (Duval-Iflah *et al.*, 1977).

Two different groups of phages which mediate the lysogenic conversion of β-lysin and staphylokinase respectively, have been reported. Simultaneous negative conversion for β-lysin expression and positive conversion for staphylokinase activity can be mediated by double-converting phages belonging to phage serotype F (Winkler *et al.*, 1965; Kondo *et al.*, 1981).

Some serotype B phages mediate positive lysogenic conversion for staphylokinase activity only (Kondo & Fujise, 1977), and other serotype A phages can convert *S. aureus* strains causing loss of β-lysin activity (Coleman *et al.*, 1986).

Reports in the literature regarding lipase conversion in *S. aureus* indicate that such phages are single-converting phages only (Rosendal & Bulow, 1965; Duval-Iflah, 1972). It is not known whether enterotoxin A-converting phages affect the expression of other *S. aureus* factors.

Previous studies from our laboratory demonstrated that β-lysin conversion by the serotype F, β-lysin and staphylokinase double-converting phage φ13, and by the serotype A, single-converting phage φ42E, was caused by insertional inactivation of the chromosomally located β-lysin determinant by the integration of phage genomic DNA during lysogen formation (Coleman *et al.*, 1986). The mechanism of *S. aureus* lipase conversion by the lipase-converting phage L54a was also shown to be due to insertional inactivation of the chromosomal lipase gene (Lee & Iandolo, 1986*a*). Positive conversion for staphylokinase and enterotoxin A expression, respectively, has been shown to be due to the location of the genes for these proteins in the DNA of converting phages, which are expressed by the lysogenic bacteria, although this has only been established for one phage in the case of staphylokinase (Sako *et al.*, 1983; Betley & Mekalanos, 1985).

Casman (1965) reported that *S. aureus* strain PS42-D, the propagating strain for the *S. aureus* typing phage 42D, carried a phage which mediated positive conversion for enterotoxin A expression. This finding was recently confirmed by Betley & Mekalanos (1985). However, Kondo & Fujise (1977) also reported that strain PS42-D carried a serotype F, β-lysin and staphylokinase double-converting phage. The possibility that these phages carried by strain PS42-D are the same or similar, and thus triple-converting, has not been investigated. Recent studies from our laboratory revealed that of nine methicillin-resistant isolates from cases of *S. aureus* septicaemia seven produced enterotoxin A ($EntA^+$) and staphylokinase (Sak^+) and all nine were β-lysin negative (Hlb^-) (Humphreys *et al.*, 1989). Since each of these properties can be affected by phage conversion, it seemed likely that these strains carried lysogenic converting phages. The present study was undertaken to establish the molecular basis for the $EntA^+$, Sak^+ and Hlb^- phenotype expressed by *S. aureus* strain PS42-D and selected Irish methicillin-resistant strains, and to determine whether these stains harboured individual converting phages which affected the expression of all of these extracellular proteins or whether more than one converting phage was involved.

METHODS

Bacterial strains, plasmids, phages and culture conditions. The bacterial strains and plasmids used are listed in Tables 1 and 2. The methicillin-resistant *S. aureus* strains DCA1, DCA2, DCA3 and DCA4 were isolated from cases of nosocomial infection in separate Irish hospitals between 1971 and 1988. Strains DCA1 (phage type 47/54/75/77/85) and DCA3 (phage type 77/84) belonged to a group of isolates with similar plasmid content and location of resistance determinants that were prevalent in Dublin hospitals between 1971 and 1975 (Coleman *et al.*, 1985). Strain DCA2 (non-typable with the International Basic Set of typing phages) belonged to another group of strains, termed Dublin Phenotype II isolates, with distinct plasmid content and location of resistance determinants that were prevalent in Dublin hospitals between 1978 and 1984 (Coleman *et al.*, 1985). Strain DCA4 (phage type 77/84) was unrelated to the other three isolates on the basis of plasmid content and antibiogram. Bacteria were routinely cultured at 37 °C for 18 h in Trypticase Soy Broth (TSB, Oxoid) for *S. aureus* strains or L-broth (LB), (Lennox, 1955) for *Escherichia coli* strains in an orbital incubator at 150 r.p.m. The corresponding agar media were also used. Brain Heart Infusion broth (BHI, Oxoid) was used for culturing *S. aureus* strains for enterotoxin A assays and as the growth medium for the *E. coli* strain DS410 and its derivatives harbouring recombinant plasmids. Sheep blood agar was prepared by incorporating packed sheep erythrocytes (5%, v/v), which had been washed three times in Tris-buffered saline (TBS; 0·14 M-NaCl, 20 mM-Tris/HCl, 10 mM-$MgSO_4$, pH 7·2), into Trypticase Soy agar (TSA, Oxoid).

Chemicals, antibiotics and enzymes. Chemicals and antibiotics were purchased from Sigma or BDH. Restriction endonucleases, T4 DNA ligase and other enzymes were purchased from Boehringer or New England Biolabs and were used according to the manufactuer's instructions. [α-^{32}P]dATP (3000 Ci mmol^{-1}; ~110 TBq mmol^{-1}) was purchased from New England Nuclear.

Bacteriophage typing. This was done by the method of Blair & Williams (1961), using the International Basic Set of typing phages for human isolates of *S. aureus*, supplied by the Central Public Health Laboratory, Colindale,

Table 1. *Bacterial strains*

Strain	Phenotype/ genotype	Comments	Source/reference
S. aureus			
RN4220	Hlb$^+$ EntA$^-$ Sak$^-$	Host strain for transformation	Kreiswirth *et al.* (1983)
DC001	Hlb$^-$ EntA$^-$ Sak$^+$	RN4220 lysogenized with ϕ13	Coleman *et al.* (1986)
80CR3	Hlb$^+$ EntA$^-$ Sak$^-$	Restriction-impaired strain	Stobberingh & Winkler (1977)
W57	Hlb$^+$ EntA$^-$ Sak$^-$	Host strain for prophages	Winkler *et al.* (1965)
PS42-D	Hlb$^-$ EntA$^+$ Sak$^+$	Propagating strain for typing phage 42D	Casman (1965)
DCA1*	Hlb$^-$ EntA$^+$ Sak$^+$	Methicillin-resistant strain, isolated 1971	This study
DCA2*	Hlb$^-$ EntA$^+$ Sak$^+$	Methicillin-resistant strain, isolated 1984	This study
DCA3*	Hlb$^-$ EntA$^+$ Sak$^+$	Methicillin-resistant strain, isolated 1975	This study
DCA4*	Hlb$^-$ EntA$^+$ Sak$^+$	Methicillin-resistant strain, isolated 1988	This study
42CR3-L	Hlb$^-$ EntA$^+$ Sak$^+$	80CR3 lysogenized with phage ϕ42	This study
A1CR3-L	Hlb$^-$ EntA$^+$ Sak$^+$	80CR3 lysogenized with phage ϕA1	This study
A3CR3-L	Hlb$^-$ EntA$^+$ Sak$^+$	80CR3 lysogenized with phage ϕA3	This study
A4CR3-L	Hlb$^-$ EntA$^+$ Sak$^+$	80CR3 lysogenized with phage ϕA4	This study
42W57-L	Hlb$^-$ EntA$^+$ Sak$^+$	W57 lysogenized with phage ϕ42	This study
42CR3-C	Hlb$^+$ EntA$^-$ Sak$^-$	42CR3-L cured of phage ϕ42	This study
A1CR3-C	Hlb$^+$ EntA$^-$ Sak$^-$	A1CR3-L cured of phage ϕA1	This study
A3CR3-C	Hlb$^+$ EntA$^-$ Sak$^-$	A3CR3-L cured of phage ϕA3	This study
A4CR3-C	Hlb$^+$ EntA$^-$ Sak$^-$	A4CR3-L cured of phage ϕA4	This study
42W57-C	Hlb$^+$ EntA$^-$ Sak$^-$	42W57-L cured of phage ϕ42	This study
DCA5	Hlb$^+$ EntA$^+$ Sak$^-$	80CR3 harbouring plasmid pDC118	This study
E. coli			
C600	*lac thr thi leu tonA supE hspR hspM*	K12 derivative	Appleyard (1954)
DS410	*ara lacY minA minB malA mtl rpsL thi*	Minicell-producing strain	Dougan & Kehoe (1984)
HB101	*recA hsdS20* ($r_B^- m_B^-$) *ara pro lac rpsL supE*	Recombination-impaired strain	Boyer & Roulland-Dussoix (1969)

* These strains were isolated from cases of nosocomial infection in Irish hospitals.

London, UK, and typing phages for bovine *S. aureus*, supplied by the Central Veterinary Laboratory, Weybridge, Surrey, UK. The bovine set consisted of phages 42D, 78, 102, 107, 116, 117, 118 and 119.

Prophage induction, prophage curing and lysogen formation. Prophages were induced from *S. aureus* strains with mitomycin C as described by de Saxe & Notley (1978). The phage-containing supernatant fractions were sterilized using 0·45 μm pore diameter membrane filters (Sartorius) and the phages propagated on *S. aureus* strain 80CR3. Then phages from several well-separated individual plaques were purified by two single-plaque isolations, using the agar layer method described by Swanstrom & Adams (1951). Lysogenization of *S. aureus* strains 80CR3 and W57 and subsequent curing of prophages from these strains by UV treatment was done as described by Coleman *et al.* (1986).

Bacteriophage serotyping. Antisera to the typing phages 6 (serotype A), 80 (serotype B) and 77 (serotype F) from the International Basic Set for typing human *S. aureus* were raised in rabbits as follows. Phage preparations (10^8 p.f.u. ml^{-1}) were prepared for injection by mixing 1 ml of phage suspension with 1 ml of complete or incomplete Freund's adjuvant. Rabbits were immunized intradermally at six dorsal sites (0·1 ml suspension per site) using antigen with complete Freund's adjuvant for primary injection followed by antigen with incomplete Freund's adjuvant for subsequent booster injections at days 10, 30 and 40. Blood was collected by cardiac puncture and the resultant serum stored at −20 °C. The method of Rountree (1949) was used to demonstrate neutralization of phage by homologous antiserum.

Large-scale purification of phage and phage genomic DNA. S. aureus strain 80CR3 was used as the propagating strain for large-scale, broth-culture phage preparations as follows. An 18 h broth culture of strain 80CR3 was diluted 100-fold into 500–1000 ml TSB broth containing 5 mM-$CaCl_2$ and shaken (150 r.p.m.) at 37 °C until the OD_{600} was about 0·2. Phage were then added to give a multiplicity of infection of 1 :10 (p.f.u. : bacterial cells). The culture was incubated at 37 °C for 20 min without shaking and then with shaking (150 r.p.m.) for 3–5 h, after which time lysis was apparently complete as evidenced by the presence of large amounts of cell debris. Phage particles were purified from bulk culture lysates using caesium chloride block gradients, following precipitation with polyethylene glycol, as described by Coleman *et al.* (1986). Genomic DNA from purified phage preparations was recovered by formamide treatment as described by Davis *et al.* (1980).

Table 2. *Plasmids*

Plasmid	Host	Resistance markers*	Comments	Source/reference
pBR322	*E. coli*	Ap^r Tc^r	Multicopy vector plasmid	Bolivar *et al.* (1977)
pAT153	*E. coli*	Ap^r Tc^r	High-copy-number variant of pBR322	Twigg & Sherratt (1980)
pACYC184	*E. coli*	Cm^r Tc^r	Multicopy vector plasmid	Chang & Cohen (1978)
pE194	*S. aureus*	Em^r	Small multicopy plasmid	Horinouchi & Weisblum (1982)
pDC020	Shuttle	Ap^r Em^r	pBR322 linked to pE194 at *Cla*I sites	Coleman *et al.* (1986)
pDC007	*E. coli*	Ap^r	pBR322 with 2·2 kb *hlb*-encoding *Hin*dIII DNA insert from *S. aureus* strain CN6708	Coleman *et al.* (1986)
pDC100	*E. coli*	Ap^r	pBR322 with 3·1 kb *sak*-encoding *Hin*dIII DNA insert from phage ϕ42	This study
pDC101	*E. coli*	Ap^r	pBR322 with 2·85 kb *sak*-encoding *Hin*dIII DNA insert from phage ϕA1	This study
pDC102	*E. coli*	Ap^r	pBR322 with 2·85 kb *sak*-encoding *Hin*dIII DNA insert from phage ϕA3	This study
pDC103	*E. coli*	Ap^r	pBR322 with 2·85 kb *sak*-encoding *Hin*dIII DNA insert from phage ϕ13	This study
pDC104	*E. coli*	Ap^r	pAT153 with 2·5 kb *entA*-encoding *Hin*dIII DNA insert from phage ϕ42	This study
pDC105	*E. coli*	Ap^r	pAT153 with 2·5 kb *entA*-encoding *Hin*dIII DNA insert from phage ϕA1	This study
pDC106	*E. coli*	Ap^r	pAT153 with 2·5 kb *entA*-encoding *Hin*dIII DNA insert from phage ϕA3	This study
pDC107	*E. coli*	Ap^r	pBR322 with 5·0 kb *attP*-encoding *Hin*dIII DNA insert from phage ϕ42	This study
pDC108	*E. coli*	Ap^r	pBR322 with 6·7 kb *attP*-encoding *Hin*dIII DNA insert from phage ϕA1	This study
pDC109	*E. coli*	Ap^r	pBR322 with 6·7 kb *attP*-encoding *Hin*dIII DNA insert from phage ϕA3	This study
pDC110	*E. coli*	Ap^r	pBR322 with 2·9 kb *attP*-encoding *Hin*dIII DNA insert from phage ϕ13	This study
pDC111	*E. coli*	Tc^r	pACYC184 with 11·3 kb *sak*- and *entA*-encoding *Eco*RI DNA insert from phage ϕ42	This study
pDC112	*E. coli*	Ap^r	pBR322 with 6·1 kb *Eco*RI–*Sal*I DNA insert from pDC111	This study
pDC113	*E. coli*	Ap^r	pBR322 with 5·2 kb *sak*- and *entA*-encoding *Eco*RI–*Sal*I DNA insert from pDC111	This study
pDC114	*E. coli*	Ap^r	pBR322 with 2·2 kb *sak*-encoding *Eco*RI–*Hin*dIII DNA insert from pDC100	This study
pDC115	*E. coli*	Ap^r	pBR322 with 2·2 kb *sak*-encoding *Eco*RI–*Hin*dIII DNA insert from pDC113	This study
pDC116	*E. coli*	Ap^r	pBR322 with 3·8 kb *attP*-encoding *Eco*RI–*Hin*dIII DNA insert from pDC107	This study
pDC117	*E. coli*	Ap^r	pBR322 with 1·2 kb *Eco*RI–*Hin*dIII DNA insert from pDC107	This study
pDC118	Shuttle	Ap^r Em^r	pDC020 with 2·5 kb *entA*-encoding *Hin*dIII DNA insert from pDC104	This study

* Abbreviations: Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance.

Isolation of plasmid and genomic DNA. Plasmid DNA from *E. coli* and *S. aureus* strains, respectively, was purified from cleared lysates of 500 ml broth cultures by centifugation in ethidium bromide/caesium chloride buoyant-density gradients as described by Clewell & Helinski (1970) and Coleman *et al.* (1986). For *E. coli* strains a rapid small-scale purification procedure from 20 ml broth cultures was also used (Coleman & Foster, 1981). Total genomic DNA from *S. aureus* strains and lysogenic derivatives was prepared from 50 ml exponential broth cultures, and purified by caesium chloride gradient centrifugation as described by Coleman *et al.* (1986). A rapid, small-scale procedure was also used as described by Mulvey *et al.* (1986).

Transformation. Transformation of plasmid DNA into $CaCl_2$-treated *E. coli* cells was done as described by Cohen *et al.* (1973). Transformation of protoplasts of *S. aureus* strain RN4220 with purified plasmid DNA was done by the method of Dowd *et al.* (1983).

Hybridization analysis. DNA probes were labelled *in vitro* by nick-translation to a specific activity $\geqslant 10^6$ d.p.m. (μg DNA)$^{-1}$ using [α-^{32}P]dATP, as described by Rigby *et al.* (1977). Restriction-enzyme-cleaved DNA was separated by electrophoresis in 0·8% or 1·0% (w/v) agarose gels, denatured, and transferred to nitrocellulose membrane filters (Schleicher & Schuell) by the method of Southern (1975). Filters were then processed under conditions of high stringency as described by Maniatis *et al.* (1982). In some experiments, filters were re-hybridized with a number of different probes. In these cases, radioactively labelled probes were removed from filters by treatment with 50 mM-NaOH as described by Anderson & Young (1985) prior to hybridization with subsequent probes.

Western immunoblotting. Polypeptides were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12·5% (w/v) gels and were electrophoretically transferred (0·5 A, 2 h) onto nitrocellulose filters (Schleicher & Schuell) as described by Burnette (1981). The *S. aureus* extracellular proteins β-lysin and enterotoxin A were detected by the method of Russell *et al.* (1985) using specific anti-β-lysin and anti-enterotoxin A sera, respectively, and a Protein A–peroxidase conjugate (20 μg; Sigma). The substrate used for visualization was 4-chloro-1-naphthol (Sigma).

Antisera. Purified *S. aureus* enterotoxin A together with the corresponding rabbit antiserum were the gifts of Professor Merlin Bergdoll (Food Research Institute, University of Wisconsin, Madison, USA). The anti-enterotoxin A serum was sequentially absorbed with concentrated suspensions of sonicated *S. aureus* strain 80CR3 and *E. coli* strain DS410 prior to use to remove non-specific antibodies. Rabbit antibodies raised against purified *S. aureus* β-lysin were supplied by Dr C. Adlam (Wellcome Research Laboratories, UK).

Enterotoxin A, β-lysin and staphylokinase assays. S. aureus strains were tested for enterotoxin A production by reverse passive latex agglutination using kits (Oxoid) according to the manufacturer's instructions, and by Western immunoblotting of culture supernatant proteins, concentrated 50-fold by ammonium sulphate precipitation (Coleman *et al.*, 1986), with anti-enterotoxin A serum. Expression of enterotoxin A by *E. coli* strain DS410 harbouring chimaeric pAT153 plasmids containing cloned fragments of *S. aureus* bacteriophage DNA was determined by colony immunoblotting with anti-enterotoxin A serum by a modification of the method described by Helfman *et al.* (1983), using a Protein A–peroxidase conjugate (20 μg, Sigma) and 4-chloro-1-naphthol as substrate. Enterotoxin A production was confirmed by Western immunoblotting of polypeptides from 50-fold concentrated culture lysates as described for *S. aureus* strains. *S. aureus* strains PS42-D and RN4220 were used as the EntA$^+$ and EntA$^-$ control strains respectively (Table 1). *E. coli* strain DS410 harbouring the cloning vector pAT153 was used as the EntA$^-$ *E. coli* strain (Tables 1 and 2).

Production of β-lysin by *S. aureus* strains, their lysogenized derivatives and derivatives cured of prophages was determined by haemolytic titration of 50-fold concentrated culture supernatant proteins and by Western immunoblotting of the concentrated preparations with specific rabbit anti-β-lysin serum, as described by Coleman *et al.* (1986).

Production of staphylokinase by *S. aureus* strains and their lysogenized and cured derivatives was tested on fibrin agar plates, with and without added canine serum 0·5% (v/v) as plasminogen source, as described by Devriese & Van de Kerckhove (1980). Staphylokinase converts plasminogen to its active form plasmin, which can degrade fibrin. Zones of clearing of the fibrin agar due to staphylokinase production should only be evident around growth of strains on fibrin agar supplemented with a source of plasminogen. The fibrin agar plates were incubated for 18–24 h at 37 °C. *S. aureus* strains DC001 and RN4220 were used as the Sak$^+$ and Sak$^-$ control strains, respectively (Table 1). Production of staphylokinase by *E. coli* derivatives harbouring chimaeric pBR322 plasmids containing cloned fragments of bacteriophage DNA was detected on fibrin agar plates supplemented with ampicillin (100 μg ml^{-1}) and canine serum (0·5%, v/v) following incubation at 37 °C for 48 h. Putative Sak$^+$ clones were further purified on fibrin agar supplemented with ampicillin and with and without added canine serum and incubated as above. *E. coli* strains C600, HB101, and DS410 harbouring pBR322 were included as Sak$^-$ controls.

Molecular cloning, restriction endonuclease cleavage site mapping and recovery of DNA fragments from agarose gels. Cloning, subcloning and restriction endonuclease site mapping experiments were done using standard techniques (Maniatis *et al.*, 1982). For cloning phage-encoded *sak* and *entA* determinants, the restriction endonuclease *Hin*dIII was used because no sites for this enzyme were found in the coding region of a *S. aureus* staphylokinase (*sak*) or enterotoxin A (*entA*) determinant, respectively, previously cloned and sequenced (Sako & Tsuchida, 1983; Betley & Mekalanos, 1988). Restriction-endonuclease-generated phage DNA fragments were recovered from low-melting-point agarose gels using Elutip-d minicolumns (Schleicher & Schuell), according to the manufacturer's instructions.

RESULTS

Screening of S. aureus for lysogenic converting phages

Five EntA$^+$, Sak$^+$ and Hlb$^-$ *S. aureus* strains were tested for carriage of lysogenic converting phages, namely PS42D and the methicillin-resistant clinical isolates DCA1, DCA2, DCA3, and

DCA4 (Table 1). Phage-containing extracts were recovered from all strains following mitomycin C induction, and phages from these preparations were separately propagated on the non-lysogenic, restriction-impaired *S. aureus* strain 80CR3 (Table 1). Phages from four randomly chosen, well-separated plaques from each lysate were single-plaque purified and further propagated on strain 80CR3, yielding a total of 20 purified phage preparations.

Properties of carried phages

Putative lysogens were isolated as non-β-haemolytic colonies on sheep blood agar from areas of confluent lysis produced by purified phage preparations on a lawn of *S. aureus* strain 80CR3. In each case, lysogeny was confirmed by demonstrating resistance to lysis by the phage used to generate lysogens and by production of phage lytic for *S. aureus* strain 80CR3 after mitomycin C induction. None of the phage isolates obtained from strain DCA2 were β-lysin converting. Four lysogens generated by separate experiments with each of the remaining 16 purified β-lysin-converting phage preparations were tested for production of staphylokinase and enterotoxin A. All 64 were Sak^+ and $EntA^+$.

Representative β-lysin converting phage isolates from each of the parental *S. aureus* strains PS42D, DCA1, DCA3 and DCA4 and a representative derivative of *S. aureus* strain 80CR3 lysogenized with each phage were chosen for detailed study. Phages were designated φ42, φA1, φA3 and φA4 and lysogens 42CR3-L, A1CR3-L, A3CR3-L and A4CR3-L (Table 1). Three independently generated, cured derivatives of each of these lysogens, prepared by UV treatment, were all found to be Hlb^+, Sak^- and $EntA^-$ (Table 1). Phage φ42, isolated from *S. aureus* strain PS42D, also converted the *S. aureus* strain W57 to the Hlb^-, Sak^+ and $EntA^+$ phenotype (Table 1). These results indicated that φ42, φA1, φA3 and φA4 were triple-converting phages, which upon lysogenization caused the simultaneous negative conversion of β-lysin and positive conversion of staphylokinase and enterotoxin A.

Serotyping triple-converting phages and phage typing of lysogens

Separate preparations of phages φ42, φA1, φA3 and φA4 were consistently neutralized by anti-serotype F serum, but not by anti-serotype A or B serum, indicating that the triple-converting phages belonged to *S. aureus* phage serotype F.

S. aureus strain 80CR3 was lysed at Routine Test Dilution (RTD) by all 23 phages of the International Basic Set of typing phages for human *S. aureus* and by the further eight phages used for typing bovine *S. aureus*. The lysogen 42CR3-L, harbouring phage φ42, was lysed only by the bovine typing phage 42D at RTD. Phage φ42 mediated a similar effect in strain 42W57-L, a lysogenic derivative of *S. aureus* strain W57. Phage φA1 blocked lysis of a lysogenic derivative of strain 80CR3 by 14 of the 31 typing phages: 11 from the International Basic Set (52, 52A, 79, 80, 55, 71, 47, 53, 83A, 95 and 96) and three from the bovine set (102, 107 and 118). Phages φA3 and φA4 did not affect the typing pattern when they lysogenized *S. aureus* strain 80CR3.

Molecular cloning of staphylokinase determinants from phages φ42, φA1, φA3 and φ13

*Hin*dIII-cleaved genomic DNA from the triple-converting phage φ42 was ligated with *Hin*dIII-cleaved vector plasmid pBR322 DNA and transformed into *E. coli* strains C600, HB101 and DS410. Transformants were selected on LB agar supplemented with ampicillin (100 μg ml^{-1}), and recombinants identified by their tetracycline-sensitive phenotype by replica-plating on LB agar supplemented with tetracycline (10 μg ml^{-1}). Fifty transformants of each of the *E. coli* host strains harbouring chimaeric plasmids were stab-inoculated into fibrin agar containing canine serum and ampicillin and incubated at 37 °C for 24 h, after which time the plates were examined for evidence of clearing of the fibrin agar around the bacterial growth. Narrow zones of clearing of the agar (2–3 mm diam.) with sharply defined edges were observed for two transformants of strain DS410. After 48 h incubation, the zones had increased substantially (7–8 mm diam.). Similar but narrower zones of clearing (2–3 mm diam.) were visible around one each of the transformants of strains HB101 and C600. All four of these transformants was stab-inoculated into fibrin agar with and without added canine serum and

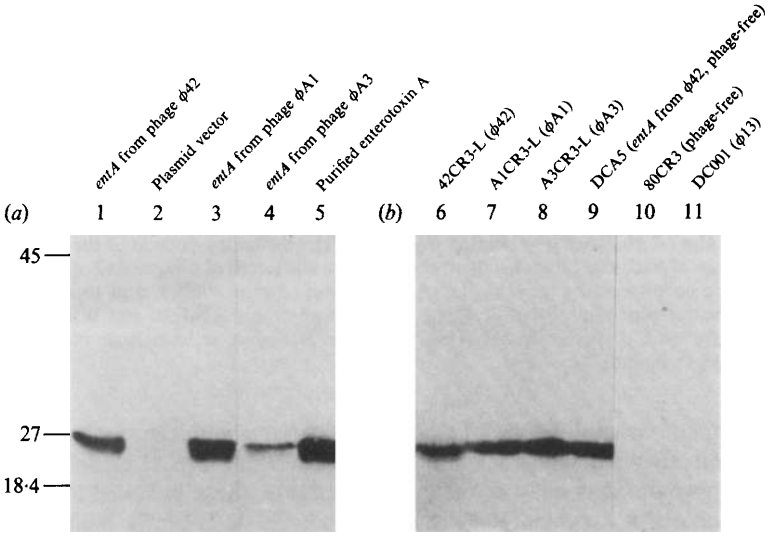


Fig. 1. Detection of enterotoxin A production by *S. aureus* strains and recombinant *E. coli* DS410 derivatives by Western immunoblotting. (*a*) Immunoblot of polypeptides expressed in *E. coli* strain DS410 derivatives harbouring recombinant *entA*$^+$ plasmids using anti-enterotoxin A serum. Phages from which *entA* determinants were cloned are shown above the track numbers. Tracks: 1, pDC104; 2, pAT153; 3, pDC105; 4, pDC106; 5, enterotoxin A purified from *S. aureus*. (*b*) Immunoblot of polypeptides from concentrated *S. aureus* culture supernatant fluids with anti-enterotoxin A serum. The strains from which material in the tracks was isolated are shown above the track numbers and phages harboured by them are shown in parenthesis. The positions of molecular mass standards are indicated: 45 kDa, ovalbumin; 27 kDa, purified *S. aureus* enterotoxin A; 18·4 kDa, β-lactoglobulin.

incubated for 48 h at 37 °C. Sharply defined zones of clearing were detected for all four recombinants only on the canine-serum-supplemented fibrin agar. After 48 h the four putative Sak$^+$ recombinants and their *E. coli* parental strains harbouring plasmid pBR322 exhibited no detectable clearing effect on the fibrin agar without added canine serum. This evidence strongly suggested that the four recombinants harboured and expressed the staphylokinase determinant (*sak*) of phage φ42. Analysis of the plasmid DNA of these four recombinants revealed that they all harboured a *Hin*dIII fragment of 3·1 kb, which corresponded in size with a *Hin*dIII-generated fragment of purified phage φ42 DNA.

Similar experiments were done with DNA from the triple-converting phages φA1 and φA3 and the double-converting phage φ13 using *E. coli* strain DS410 as the host strain for transformation. Sak$^+$ recombinants were recovered from each of these phages at a frequency of approximately one per fifty recombinants. Analysis of the plasmid DNA from four independently generated Sak$^+$ recombinants in each case revealed that all 12 harboured a *Hin*dIII fragment of 2·85 kb and in all cases the fragments corresponded in size to a *Hin*dIII-generated fragment of the respective parental phage genomic DNAs. Representative chimaeric plasmids harbouring the cloned *sak* determinant from each of the triple-converting phages φ42, φA1 and φA3 and the double converting phage φ13 were chosen for further study (Table 2).

Molecular cloning of enterotoxin A determinants from phages φ42, φA1 and φA3

Approximately 100 transformants of *E. coli* strain DS410, harbouring recombinant derivatives of the cloning vector pAT153 containing cloned *Hin*dIII fragments of the triple-converting phage φ42, were screened for expression of enterotoxin A by colony immunoblotting with specific anti-enterotoxin A serum. Three putative EntA$^+$ recombinants were tested for expression of enterotoxin A by Western immunoblotting of 50-fold concentrated cell lysates with anti-enterotoxin A serum. In each case a single polypeptide of 27 kDa was detected, which corresponded in molecular mass to purified staphylococcal enterotoxin A (Fig. 1, tracks 1 and 5).

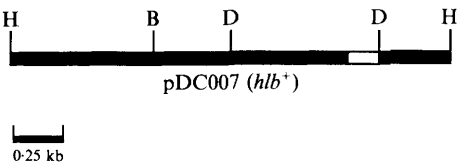


Fig. 2. Restriction map of the 2·2 kb *Hin*dIII fragment cloned in plasmid pDC007 which encodes the cloned *S. aureus* β-lysin determinant (*hlb*), and which was used as the source of the 0·75 kb *Dde*I-generated and the 2·2 kb *Hin*dIII-generated *hlb* probes. The unshaded portion of the figure refers to a 0·15 kb sequence of bacterial DNA to which the insertion site (*attB*) of phages φ42, φA1 and φA3 was localized in the corresponding genomic DNA of *S. aureus* strain 80CR3, and to a similar 0·15 kb sequence in the corresponding genomic DNA of *S. aureus* strains RN4220 and W57 in the cases of phages φ13 and φ42, respectively. Abbreviations for restriction endonuclease cleavage sites: H, *Hin*dIII; D, *Dde*I; B, *Bcl*I.

Similar experiments were done with DNA from the triple-converting phages φA1 and φA3 and the double-converting phage φ13. In the case of the former, EntA$^+$ clones were recovered at a frequency of approximately one per forty recombinants; these behaved like the φ42 EntA$^+$ clones in immunoblotting experiments (Fig. 1, tracks 3 and 4; Table 2). No EntA$^+$ clones were detected among recombinants generated from genomic DNA of phage φ13, even when 300 recombinants generated from eight separate cloning experiments were screened.

Analysis of the plasmid DNA of representative EntA$^+$ transformants revealed that each harboured a single *Hin*dIII insert of 2·5 kb which corresponded in size to a single *Hin*dIII fragment detected in native phage genomic DNAs. Representative chimaeric plasmids harbouring the cloned enterotoxin A determinants (*entA*) of phages φ42, φA1 and φA3 were chosen for further study (Table 2). No detectable homology was observed when *Hin*dIII-cleaved phage φ13 DNA was probed with the 2·5 kb *entA*-containing *Hin*dIII fragment of phage φ42, indicating that phage φ13 did not encode an *entA* determinant (Fig. 3, panel 7, track 2).

Expression of cloned sak and entA determinants in S. aureus

The cloned *Hin*dIII fragments encoding the *sak* and *entA* determinants, respectively, of phages φ42, φA1, φA3 and φ13 were subcloned into the single *Hin*dIII site of the shuttle vector pDC020 (Table 2), which is capable of replicating in *E. coli* and *S. aureus*, and transformed separately into protoplasts of *S. aureus* strain RN4220. Transformants were selected on agar containing 10 μg erythromycin ml^{-1} and purified on agar containing 50 μg erythromycin ml^{-1}. Twenty transformants in each case, which contained the cloned *sak* determinants of the phages, were stab-inoculated into fibrin agar containing erythromycin (50 μg ml^{-1}) with or without canine serum. Following incubation at 37 °C for 18 h, sharply defined zones of fibrin clearing (3–5 mm diam.) on canine-serum-supplemented agar were observed around the vast majority of transformants, but not on the agar without canine serum, even after 48 h incubation. Transformants of strain RN4220 harbouring only the shuttle vector were Sak$^-$ on both types of media.

Five RN4220 transformants each, respectively, harbouring shuttle plasmid derivatives containing the cloned *entA*-encoding *Hin*dIII fragments of phages φ42, φA1 and φA3 were EntA$^+$ when tested by reverse passive latex agglutination assay. Transformants harbouring the shuttle plasmid pDC020 only were EntA$^-$. These results were confirmed for two representative transformants in each case by immunoblotting of concentrated culture supernatant proteins with anti-enterotoxin A serum. A single polypeptide of 27 kDa, corresponding in molecular mass to purified enterotoxin A from *S. aureus*, was detected in each case. An example is shown in Fig. 1, track 9.

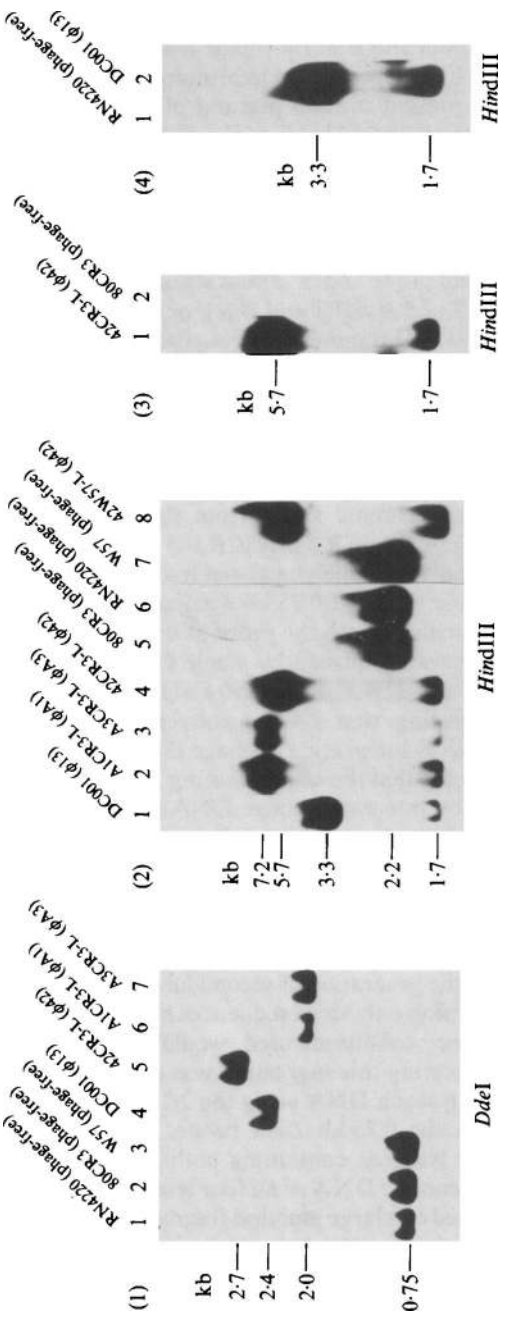
Mechanism of negative β-lysin conversion mediated by triple-converting phages

To determine the mechanism of negative β-lysin conversion of *S. aureus* strain 80CR3 by triple-converting phages, it was necessary to compare the structure of the β-lysin determinant (*hlb*) of strain 80CR3 with its lysogenic derivatives.

The 2·2 kb *Hin*dIII fragment of plasmid pDC007 (Table 2; Fig. 2), which harboured the cloned *hlb* determinant of *S. aureus* strain CN6708 (Coleman *et al.*, 1986), was used as the source of a *hlb* probe. The 0·75 kb internal *Dde*I segment of DNA encoding most of the *hlb* structural determinant from pDC007 was used to probe genomic DNA of *S. aureus* strain 80CR3 cleaved separately with *Dde*I and *Hin*dIII. Single *Dde*I (Fig. 3, panel 1, track 2) and *Hin*dIII fragments of 0·75 kb and 2·2 kb, respectively, hybridized with the probe, indicating that homologous *hlb* sequences were present in both plasmid pDC007 and *S. aureus* strain 80CR3. Hybridization experiments with the 0·75 kb *hlb* probe and strain 80CR3 DNA digested separately with the restriction enzymes *Cla*I and *Eco*RI, which do not cleave within the *hlb* determinant (Coleman *et al.*, 1986), detected the presence of single homologous fragments in each case, respectively (data not shown), indicating that a single copy of the *hlb* determinant was present in strain *S. aureus* 80CR3. Additional hybridization experiments with the 2·2 kb *Hin*dIII fragment of pDC007 as the *hlb* probe and *S. aureus* strain 80CR3 DNA cleaved with (i) *Dde*I, (ii) *Dde*I and *Hin*dIII, (iii) *Bcl*I and *Hin*dIII and *Dde*I, or (iv) *Hin*dIII and *Bcl*I demonstrated that the 2·2 kb *hlb*-containing *Hin*dIII fragment of *S. aureus* strain 80CR3 had a similar structure to that cloned in plasmid pDC007 from strain CN6708 (Fig. 2). Similar experiments established that *S. aureus* strain W57 harboured a single copy of the *hlb* determinant, encoded on a 2·2 kb *Hin*dIII fragment, which was homologous with and had a structure similar to that of plasmid pDC007 and *S. aureus* strain 80CR3 (Fig. 2; Fig. 3, panel 1, track 3, and panel 2, track 7).

Hybridization studies were then performed using the 0·75 kb *hlb* probe with *Hin*dIII-cleaved and *Dde*I-cleaved genomic DNA from the pairs of *S. aureus* strains 42CR3-L/42CR3-C, A1CR3-L/A1CR3-C, A3CR3-L/A3CR3-C and 42W57-L/42W57-C, derivatives of *S. aureus* strains 80CR3 and W57 which had been lysogenized with and cured of triple converting phages respectively (Table 1). Single 0·75 kb *Dde*I and 2·2 kb *Hin*dIII fragments, respectively, from the cured strains hybridized with the probe as occurred previously with the parental strains 80CR3 and W57. These were replaced by single fragments of 2·7 kb (*Dde*I) and 5·7 kb (*Hin*dIII) in strains 42CR3-L and 42W57-L, and 2·0 kb (*Dde*I) and 7·25 kb (*Hin*dIII) in strains A1CR3-L and A3CR3-L, suggesting that β-lysin conversion in these lysogens occurred by insertional inactivation of *hlb* by integration of phage DNA (Fig. 3, panel 1, tracks 5–7). If this was the case, it would be expected that the *hlb*-containing *Dde*I and *Hin*dIII fragments would be split into two parts separated by integrated phage DNA sequences. Furthermore, since each of the phages φ42, φA1 and φA3 had several *Hin*dIII and *Dde*I restriction sites (data not shown), the 0·75 kb *hlb* probe should have identified two junction fragments in both the *Dde*I- and *Hin*dIII-cleaved lysogen DNA, composed partly of phage DNA and partly of the *hlb*-containing fragments in each case. Failure to detect the second junction fragment could have been due to the location of the phage insertion site(s) near one end of the 0·75 kb *hlb*-containing *Dde*I fragment. This would have resulted in the generation of second junction fragments composed predominantly of phage DNA and containing only short sequences homologous with the 0·75 kb *hlb* probe, which under the high stringency conditions used, would not hybridize effectively with the probe.

Evidence supporting this suggestion was obtained following hybridization experiments with *Hin*dIII-cleaved lysogen DNA using the 2·2 kb pDC007-derived *hlb* probe. Because this probe was longer than the 0·75 kb *Dde*I probe, it was likely that it would overlap the genomic sequences of the lysogens containing both junction fragments. Two junction fragments were detected in the genomic DNA of all four lysogens (42CR3-L, A1CR3-L, A3CR3-L and 42W57-L). These consisted of a large junction fragment, corresponding in size to those detected with the 0·75 kb *hlb* probe in *Hin*dIII-cleaved lysogen DNA, and a small junction fragment of 1·7 kb in each case (Fig. 3, panel 2, tracks 2, 3, 4 and 8). The small junction fragments were consistently detected as weaker signals compared with the large junction fragments, suggesting that the insertion site(s) for the three triple-converting phages tested was (were) located near to one end of the *hlb*-containing *Hin*dIII fragment in the bacterial genomic DNA. These experiments were repeated with DNA from three independently generated lysogenic derivatives of *S. aureus* strain 80CR3 for each of the phages φ42, φA1 and φA3 and with DNA from an additional three lysogenic derivatives of *S. aureus* strain W57 harbouring phage φ42. Results indentical to those described above were obtained in each case.


(1)
DdeI
kb
2·7
2·4
2·0
0·75
1 RN4220 (phage-free)
2 80CR3 (phage-free)
3 W57 (phage-free)
4 DC001 (ϕ13)
5 42CR3-L (ϕ42)
6 A1CR3-L (ϕA1)
7 A3CR3-L (ϕA3)
(2)
HindIII
kb
7·2
5·7
3·3
2·2
1·7
1 DC001 (ϕ13)
2 A1CR3-L (ϕA1)
3 A3CR3-L (ϕA3)
4 42CR3-L (ϕ42)
5 80CR3 (phage-free)
6 RN4220 (phage-free)
7 W57 (phage-free)
8 42W57-L (ϕ42)
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1 42CR3-L (ϕ42)
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1 RN4220 (phage-free)
2 DC001 (ϕ13)

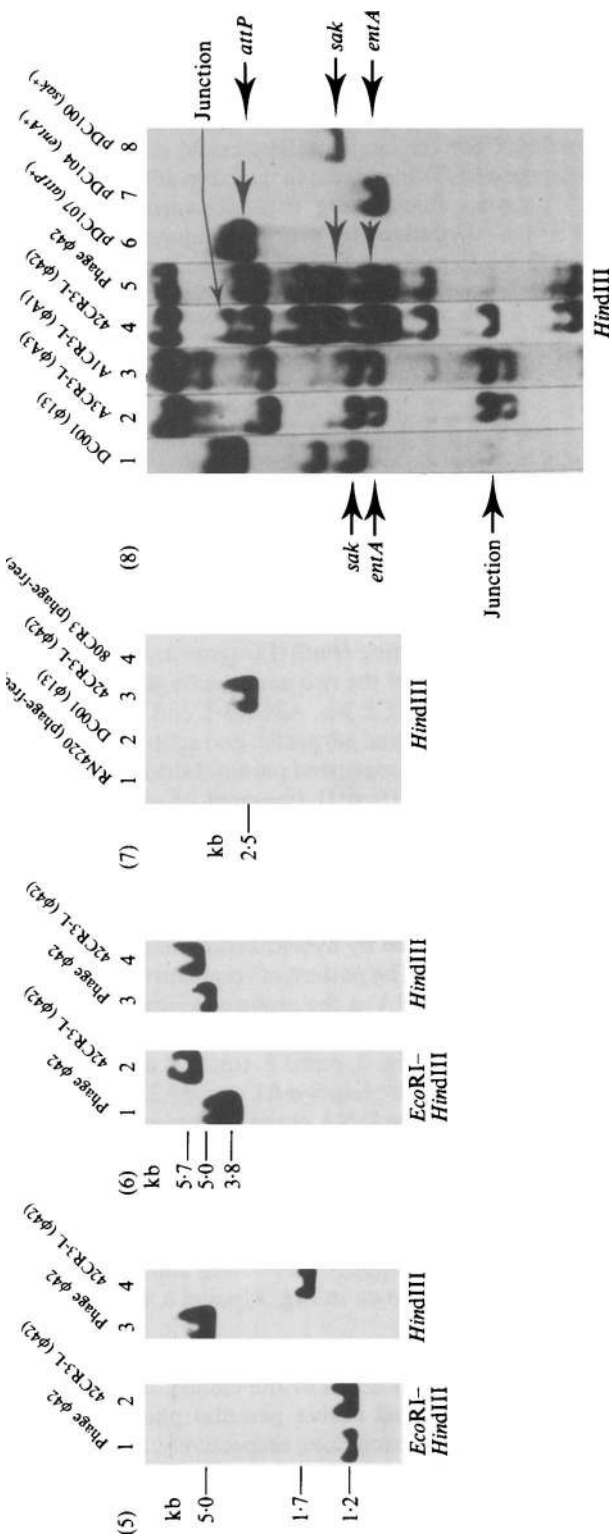

Fig. 3. Southern hybridization analysis of restriction-endonuclease-digested chromosomal DNA of *S. aureus* strains and lysogenized derivatives. The source of DNA in each track is shown above the track numbers of each panel; phages harboured by individual *S. aureus* strains are shown in parenthesis. The restriction endonucleases used to cleave DNA are shown beneath each panel. The provenance of recombinant plasmids which were used as the source of molecular probes is shown in Table 2 and Fig. 4(*b*). DNA was hybridized with probes as follows: panel 1, 0·75 kb *Dde*I *hlb* fragment of pDC007; panel 2, 2·2 kb *Hin*dIII *hlb* fragment of pDC007; panel 3, 5·0 kb *Hin*dIII *attP* fragment of ϕ42 from plasmid pDC107; panel 4, 2·9 kb *Hin*dIII *attP* fragment of ϕ13 from plasmid pDC110; panel 5, 1·2 kb *Eco*RI–*Hin*dIII fragment of plasmid pDC117; panel 6, 3·8 kb *Eco*RI–*Hin*dIII fragment of plasmid pDC116; panel 7, 2·5 kb *Hin*dIII *entA* fragment of ϕ42 from plasmid pDC104; panel 8, phage ϕ42 genomic DNA. In panel 8, the position of the small *Hin*dIII junction fragments of the lysogens (tracks 1–4) is shown on the left, as are the *Hin*dIII fragments encoding *sak* and *entA*, respectively, of lysogens DC001, A1CR3-L and A3CR3-L (tracks 1–3); the large *Hin*dIII junction fragment of lysogen 42CR3-L (track 4), the *Hin*dIII fragments encoding *sak* and *entA*, respectively, of lysogen 42CR3-L and phage ϕ42 (tracks 4 and 5), and the *attP*-containing *Hin*dIII fragment of ϕ42 (track 5) are indicated on the right.

Similar results have previously been reported from this laboratory in regard to β-lysin conversion of *S. aureus* strain RN4220, mediated by the β-lysin and staphylokinase double-converting phage φ13 (Coleman *et al.*, 1986). These studies also demonstrated that the *hlb* determinant of RN4220 was located on a 2·2 kb *Hin*dIII fragment indistinguishable from that cloned in plasmid pDC007 (Fig. 2). To directly compare, in the same experiment, the mechanisms of double and triple phage-mediated conversion, *Hin*dIII-cleaved genomic DNA of strain DC001 (RN4220 lysogenized with phage φ13, Table 1) was included in adjacent tracks to *Hin*dIII-cleaved DNA of strain 80CR3 lysogens (harbouring triple-converting phages) in hybridization experiments using the 2·2 kb pDC007-derived *hlb* probe. Two junction fragments were detected following hybridization with DNA from strain DC001 : a large junction fragment of 3·3 kb and a weaker, small junction fragment of 1·7 kb indistinguishable in size from that detected with the strain 80CR3 lysogens (Fig. 3, panel 2, track 1). Identical results were obtained with DNA from three additional, independently generated, lysogenic derivatives of strain RN4220 harbouring phage φ13.

Cloning of phage attachment sites

If the two junction fragments identified in the *Hin*dIII-cleaved lysogen DNA using the 2·2 kb *hlb* probe were generated by the integration of phage DNA sequences, similar hybridization experiments using cloned *Hin*dIII fragments containing phage attachment sites (*attP*) as molecular probes, should allow the detection of the same two junction fragments in each case, respectively.

An approximate estimate of the sizes of the *attP*-containing *Hin*dIII fragments of phages φ42, φA1, φA3 and φ13 was obtained by combining the sizes of the two junction fragments detected in the *Hin*dIII-cleaved DNA of the lysogens 42CR3-L, A1CR3-L, A3CR3-L and DC001 (Table 1 ; Fig. 3, panel 2, tracks 1–4) with the 2·2 kb pDC007-derived *hlb* probe, and subtracting the size of the *hlb*-containing *Hin*dIII fragment (2·2 kb) of the unlysogenized parental strains 80CR3 and RN4220, respectively. The size of the *attP*-containing *Hin*dIII fragment of phage φ42 was estimated as 5·2 kb, whereas those of phages φA1, φA3 and φ13 were estimated to be 6·75, 6·75 and 2·8 kb, respectively.

In order to identify the *attP*-containing *Hin*dIII fragments of phages φ42, φA1, φA3 and φ13, *Hin*dIII-cleaved DNA of these phages and genomic DNA of the corresponding lysogens 42CR3-L, A1CR3-L, A3CR3-L and DC001 was examined by hybridization analysis using the native phage genomes in each case as molecular probes. The pattern of fragments detected with both phage φ42 and 42CR3-L DNA using phage φ42 DNA as the probe was similar, but a φ42 DNA fragment of approximately 5·0 kb was absent in the DNA of the φ42 lysogen 42CR3-L, and replaced by a single detectable fragment of 5·7 kb (Fig. 3, panel 8, tracks 4 and 5). *Hin*dIII fragments of 6·7 kb detected in the digested DNA of both phages φA1 and φA3, and of 2·8 kb detected with digested phage φ13 DNA, were absent in the DNA of the corresponding lysogens and replaced by fragments of 7·25 kb in the case of phages φA1 and φA3, and of 3·3 kb in the case of phage φ13. The larger fragments detected in the lysogen DNAs corresponded in size to the large junction fragments detected in hybridization experiments with *hlb* probes. Failure to identify the expected 1·7 kb second junction fragment in the lysogen DNAs was probably due to masking by other phage DNA fragments, as all the native phage DNAs had *Hin*dIII fragments of approximately 1·7 kb. An example of these results is shown in Fig. 3 (panel 8, tracks 4 and 5) in respect of phage φ42.

The *attP*-containing *Hin*dIII fragments of phages φ42, φA1, φA3 and φ13 were cloned into pBR322 following extraction from agarose gels (Table 2). Each of the cloned *attP*-containing *Hin*dIII fragments was used to probe its *Hin*dIII-cleaved native parental phage DNA and corresponding *S. aureus* strain 80CR3 lysogen DNA. In each case, respectively, single *Hin*dIII fragments corresponding in size to the *attP* probes were detected with the digested phage DNA. The *attP* probes hybridized with two fragments in the lysogen DNAs, respectively, which corresponded in size to the junction fragments detected previously using the 2·2 kb pDC007-derived *hlb* probe (Fig. 3, panel 3, track 1, and panel 4, track 2). No homology was detected with

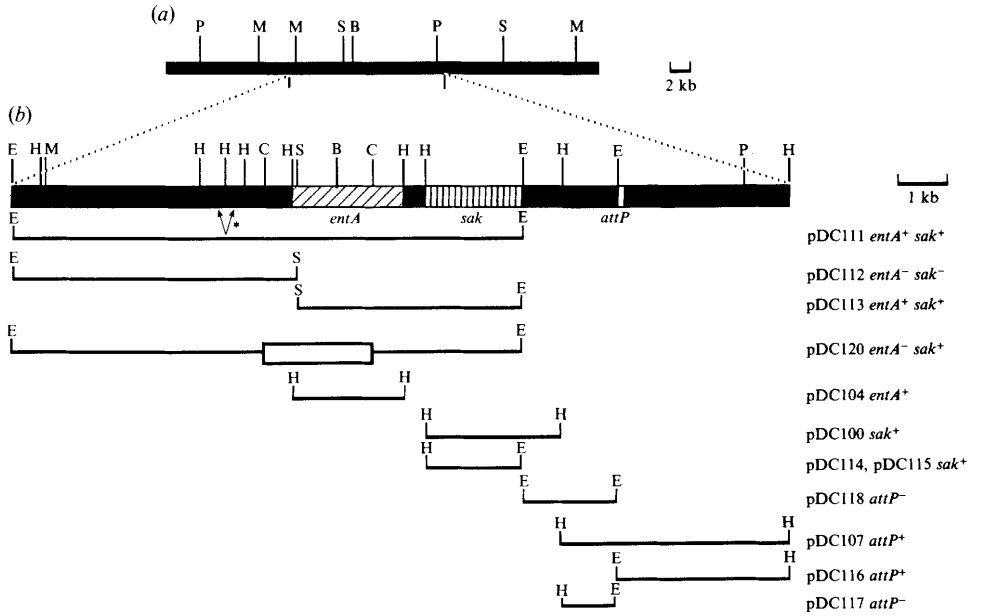

Fig. 4. (*a*) Restriction endonuclease cleavage site map of linear phage ϕ42 genomic DNA. The black rectangular box represents the linear phage ϕ42 genome. (*b*) Restriction endonuclease cleavage site map of a 17·4 kb DNA region from within the phage ϕ42 genome, which is represented by the large rectangular box. The dotted diagonal lines indicate the portion of phage ϕ42 DNA which was mapped in detail. The DNA sequences to which the *entA* (diagonal shading) and *sak* (vertical shading) determinants were localized are indicated. The small unshaded area of the rectangular box represents a 0·15 kb DNA sequence to which *attP* was localized. The thin horizontal lines in the lower portion of the figure refer to sequences of DNA which were cloned in the corresponding plasmids listed to the right. Plasmid pDC120 was derived from pDC111 by deletion of the DNA sequences between the *Cla*I sites of the cloned DNA of pDC111, and this deletion is represented by an open rectangular box. Abbreviations: P, *Pst*I; M, *Mlu*I; S, *Sal*I; B, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; C, *Cla*I. *The exact juxtaposition of the two small *Hin*dIII fragments indicated was not established.

DNA of *S. aureus* strains 80CR3 or RN4220 and any of the phage DNA or *attP*-fragment probes (Fig. 3, panel 3, track 2, and panel 4, track 1).

These results confirmed that the mechanism of β-lysin conversion mediated by the triple-converting phages was due to insertional inactivation of the *hlb* determinant by integration of phage DNA, and that the mechanism was very similar to that mediated by the double-converting phage ϕ13.

Organization of sak, entA and attP sequences in phage genomic DNA

A restriction map of phage ϕ42 DNA was generated using the restriction enzymes *Pst*I, *Mlu*I, *Sal*I and *Bgl*I (Fig. 4a). Following agarose gel electrophoresis of duplicate samples of *Mlu*I-cleaved phage ϕ42 DNA, one sample of which had been incubated at 70 °C for 10 min, a fragment of 13 kb which was present in the unheated phage DNA digest was markedly reduced in intensity in the corresponding heated sample, with a concomitant increase in the intensity of appearance of two bands of 10·5 and 2·5 kb, respectively. The latter fragments corresponded in size to the two *Mlu*I-generated end fragments of phage ϕ42 DNA (Fig. 4*a*). This evidence indicated that the genome of phage ϕ42 consisted of a linear DNA molecule with cohesive ends which could anneal to form a circular molecule. The approximate location of the phage ϕ42 *entA*, *sak* and *attP* determinants was established following hybridization experiments with phage ϕ42 DNA co-digested with *Mlu*I and *Sal*I (Fig. 4*a*) and using the excised phage ϕ42 *sak*-, *entA*- and *attP-Hin*dIII fragments cloned in plasmids pDC100, pDC104 and pDC107 as molecular probes (Table 2). In separate experiments, all three probes hybridized with the central

18 kb *Sal*I fragment of phage ϕ42 DNA, indicating that both the *entA* and *sak* determinants and the *attP* site were located within this fragment (Fig. 4*a*).

Additional cloning experiments were done in order to localize these determinants. *Eco*RI fragments of phage ϕ42 DNA were cloned into the plasmid vector pACYC184 and transformed into *E. coli* strain HB101. One of these transformants harboured an *Eco*RI DNA insert of 11·3 kb and mediated the expression of staphylokinase and enterotoxin A activity, although weakly; the recombinant plasmid was termed pDC111 (Table 2; Fig. 4*b*). Attempts to transform pDC111 into *E. coli* strain DS410 failed, probably due to instability of the DNA insert. A 2·5 kb *Hin*dIII fragment located centrally in the cloned DNA of plasmid pDC111 corresponded in size and structure to the *entA*-containing *Hin*dIII fragment originally cloned in pDC104 (Fig. 4*b*; Table 2).

The 6·12 kb and 5·17 kb *Sal*I–*Eco*RI fragments of pDC111 were subcloned into pBR322 and the recombinant plasmids termed pDC112 and pDC113 respectively (Fig. 4*b*; Table 2). Derivatives of *E. coli* strain DS410 harbouring pDC113 were EntA$^+$ and Sak$^+$. The *sak* determinant was further localized by subcloning the 2·2 kb *Eco*RI–*Hin*dIII fragment of pDC113 into pBR322 yielding the *sak*$^+$ plasmid pDC115 (Fig. 4*b*). These results were confirmed by subcloning the 2·2 kb *Eco*RI–*Hin*dIII fragment of the *sak*$^+$ plasmid pDC100 into pBR322, yielding the *sak*$^+$ plasmid pDC114 (Fig. 4*b*). Additional cloning, mapping and hybridization experiments revealed that the *attP*-containing 5·0 kb *Hin*dIII fragment of phage ϕ42, cloned in plasmid pDC107, was located to the right of the *sak* determinant as drawn in Fig. 4(*b*).

The position of the ϕ42 *attP* site was further localized by using the cloned inserts of plasmids pDC116 and pDC117 as probes (Fig. 4*b*; Table 2). The 1·2 kb *Eco*RI–*Hin*dIII insert of plasmid pDC117 detected corresponding fragments of 1·2 kb in *Eco*RI–*Hin*dIII-cleaved ϕ42 DNA and in strain 42CR3-L DNA (Fig. 3, panel 5, tracks 1 and 2). This probe also hybridized with the 5·0 kb *attP*-containing *Hin*dIII fragment in *Hin*dIII-cleaved ϕ42 DNA and with a fragment of 1·7 kb, corresponding to the small junction fragment previously detected with *hlb* probes, in *Hin*dIII-cleaved strain 42CR3-L DNA (Fig. 3, panel 5, tracks 3 and 4). These results demonstrated that the *attP* site of phage ϕ42 was located to the right of the internal *Eco*RI site of the 5·0 kb *attP*-containing *Hin*dIII fragment as drawn in Fig. 4(*b*). Confirmation of these findings was obtained by using the 3·8 kb *Eco*RI–*Hin*dIII insert of plasmid pDC116 to probe *Hin*dIII-cleaved and *Eco*RI–*Hin*dIII-cleaved ϕ42 and strain 42CR3-L DNA (Fig. 3, panel 6, tracks 1–4).

A more precise location for *attP* was deduced by calculating the relative proportions of phage and bacterial DNA which comprised the large and small *Hin*dIII junction fragments of the phage ϕ42 lysogen 42CR3-L. Since the 1·2 kb *Eco*RI–*Hin*dIII fragment cloned in plasmid pDC117 (Fig. 4*b*) hybridized with a corresponding fragment in *Eco*RI–*Hin*dIII-cleaved lysogen 42CR3-L DNA but with the 1·7 kb *Hin*dIII small junction fragment in *Hin*dIII-cleaved lysogen 42CR3-L DNA, the small junction fragment must have consisted of (i) 1·2 kb of phage ϕ42 DNA corresponding to the DNA of the probe, (ii) phage DNA sequences located to the right of the 1·2 kb *Eco*RI–*Hin*dIII fragment harboured by plasmid pDC117 (Fig. 4*b*), and (iii) DNA sequences from the chromosomally located 2·2 kb *hlb*-containing *Hin*dIII fragment of the *S. aureus* parental strain 80CR3 (Fig. 2; Fig. 4*b*). Thus, DNA sequences from (ii) and (iii) above would only have contributed 0·5 kb to the size of the small *Hin*dIII junction fragment, indicating that the *attP* site was located close to the internal *Eco*RI site of the *attP*-containing *Hin*dIII fragment (Fig. 4*b*). Furthermore, since the chromosomal insertion site of phage ϕ42 was positioned in the 0·75 kb *Dde*I fragment of the 2·2 kb *hlb*-containing *Hin*dIII fragment of *S. aureus* strain 80CR3, one of the two *Dde*I–*Hin*dIII fragments of 1·1 kb and 0·35 kb, respectively, flanking the 0·75 kb *Dde*I fragment must also have formed part of the small *Hin*dIII junction fragment in the DNA of the lysogen (Fig. 2). Seeing that only 0·5 kb of the small junction fragment remained unaccounted for, the 0·35 kb *Dde*I–*Hin*dIII fragment flanking the chromosomal phage insertion site must have formed part of the small junction fragment in the corresponding DNA of the lysogen. The remaining 0·15 kb of the small *Hin*dIII junction fragment must have consisted of part of the 0·75 kb *hlb*-containing *Dde*I fragment of *S. aureus* strain 80CR3 (Fig. 2) and part of the DNA of phage ϕ42 to the right of the *Eco*RI site within the *attP*-containing *Hin*dIII fragment of phage ϕ42, as drawn in Fig. 4(*b*).

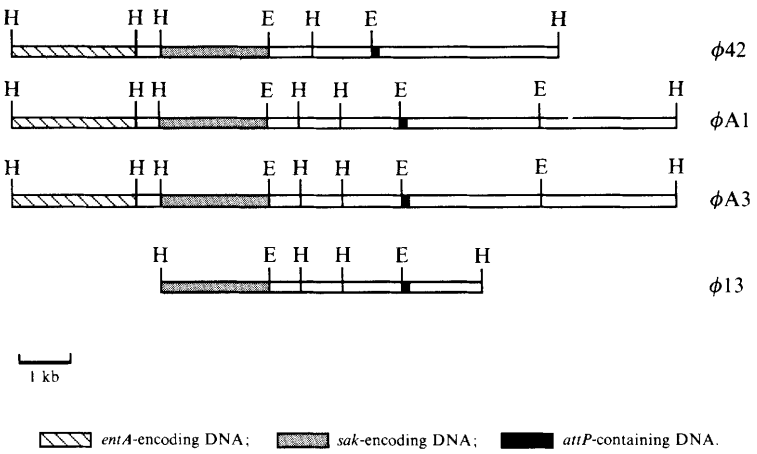

Fig. 5. *Eco*RI and *Hin*dIII cleavage site maps of the *entA*-, *sak*- and *attP*-containing DNA regions of the triple-converting phages φ42, φA1 and φA3 and the *sak*- and *attP*-containing DNA region of the double-converting phage φ13. The 2·5 kb *Hin*dIII fragments to which the *entA* determinants were localized and the 2·2 kb *Eco*RI–*Hin*dIII fragments to which the *sak* determinants were localized are indicated. For each phage *attP* was localized to a 0·15 kb region of DNA.

Similar experiments were done to map the *entA*-, *sak*- and *attP*-containing sequences of phages φA1, φA3 and φ13 (Fig. 5), all of which were found to have cohesive termini. The position of the *Hin*dIII site to the right of *attP* (as drawn in Fig. 5) varied for three of the phages φ42, φA1, φA3 and φ13, accounting for the differences in the sizes of the large junction fragments detected with *hlb* probes in *Hin*dIII-cleaved lysogen DNA (Fig. 5).

Hybridization analysis of *Hin*dIII-cleaved DNA of phages φA1, φA3 and φ13 using phage φ42 DNA as a probe demonstrated that phages φA1, φA3 and φ13 shared some homology with phage φ42, but the homology detected was considerably less than that detected between phage φ42 and the probe (Fig. 3, panel 8, tracks 1–5). These results indicated that phages φA1, φA3 and φ13 were more distantly related to phage φ42 than was initially apparent on the basis of similarities in their respective DNA regions encoding *entA*, *sak* and *attP*.

DISCUSSION

Since Winkler *et al.* (1965) first described the concomitant lysogenic conversion of *S. aureus* β-lysin and staphylokinase, it has been well established that this phenomenon is invariably mediated by serotype F double-converting phages (Kondo & Fujise, 1977; Kondo *et al.*, 1981). This paper reports the discovery and molecular analysis of a novel group of *S. aureus* serotype F phages, which mediate the simultaneous triple-lysogenic conversion of enterotoxin A, staphylokinase and β-lysin. Three of these phages were recovered from methicillin-resistant strains isolated in Irish hospitals between 1971 and 1988, and the fourth from strain PS42-D, which has been used as the propagating strain for the *S. aureus* typing phage 42D since before 1965 (Casman, 1965). This evidence suggests that the triple-converting phages have not evolved in the last twenty years and probably existed when Winkler *et al.* (1965) first described the double-converting phages. In support of this suggestion, Casman (1965) reported the recovery of an enterotoxin A converting phage from strain PS42-D, although he did not report whether this phage affected the conversion of other *S. aureus* characters. Also Kondo & Fujise (1977) described the isolation of a serotype F β-lysin and staphylokinase converting phage from *S. aureus* strain PS42-D, but did not report whether this phage affected the conversion of enterotoxin A. It is possible, in view of the present study, that the phages described by both of these reports were in fact triple-converting.

The triple-converting phages φ42, φA1 and φA3 were chosen for detailed molecular analysis because they could be differentiated on the basis of their effect on the phage typing pattern of *S.*

aureus strain 80CR3 upon lysogenization. In each case the *entA* and *sak* determinants of these phages were linked in the phage DNA in close proximity to the phage attachment site (*attP*) and furthermore, the *sak* determinant of the double-converting phage ϕ13 was also encoded close to its *attP* site. Analysis of several additional serotype F β-lysin and staphylokinase converting phages from clinical and bovine *S. aureus* strains revealed a similar situation with the *sak* determinants located adjacent to *attP* (D. C. Coleman, unpublished data). This evidence suggests that the triple- and double-converting phages at some time in the past may have acquired their *entA* and *sak* determinants, respectively, by imprecise excision events between chromosomally located *entA* and *sak* determinants and closely inserted prophages, and that these determinants have now become integral to the phage DNA. Similar mechanisms have been proposed to explain the findings that corynephage β and the streptococcal phage T12 carry the genes for diphtheria toxin and streptococcal pyrogenic exotoxin A very close to their respective *attP* sites (Laird & Groman, 1976; Johnson *et al.*, 1986).

The restriction maps of the *entA*-, *sak*- and *attP*-DNA regions of phages ϕ42, ϕA1, ϕA3 and ϕ13 were remarkably similar, suggesting a close relationship. However, hybridization analysis using phage ϕ42 genomic DNA as a molecular probe indicated that phages ϕA1, ϕA3 and ϕ13 were distantly related to ϕ42. These findings are consistent with a recent report by Inglis *et al.* (1987) which demonstrated that some serotype F *S. aureus* phages share less than 50% sequence homology. Whether phage ϕ13 was originally derived from a triple-converting phage or acquired its *sak* determinant independently by an imprecise excision event is speculative.

The triple-converting phage ϕ42 dramatically altered the phage typing pattern of the *S. aureus* strains 80CR3 and W57 upon lysogenization. Phage ϕA1 had a similar but less extensive effect upon lysogenization of *S. aureus* 80CR3, whereas the triple-converting phages ϕA3 and ϕA4 did not affect the phage typing pattern at all. Similar observations concerning the narrowing of the phage typing pattern of *S. aureus* strains upon lysogenization by a range of bacteriophages have been reported previously (Rountree, 1959; Vickery *et al.*, 1986). The basis for this phenomenon is not yet understood. However, the differences in the extent of narrowing of the phage typing pattern of *S. aureus* by three of the triple-converting phages suggests that although the phages belong to the same serotype and have other features in common, they are not identical.

The properties of the triple-converting phage ϕ42, which was recovered from *S. aureus* strain PS42-D, are very similar to those of the enterotoxin A converting phage PS42-D described by Betley & Mekalanos (1985), which was also recovered from a culture of strain PS42-D. However, these authors did not report whether their phage affected the expression of staphylokinase or β-lysin. The restriction enzyme cleavage site maps of the two phages are very similar; both phages encoded *entA* near to the *attP* site and both had linear genomes with cohesive termini. However, there is one major difference between the two studies. Betley & Mekalanos (1985) reported that the phage-free *S. aureus* strain 80CR3 when lysogenized with phage PS42-D contained integrated prophage DNA and linear phage DNA. In the present study, several independent derivatives of *S. aureus* strain 80CR3 lysogenized with phage ϕ42 contained integrated prophage DNA only. Similar results were obtained with derivatives of *S. aureus* strain W57 lysogenized with phage ϕ42. These findings suggest that the lysogens generated by Betley & Mekalanos (1985) with phage PS42-D were unstable and that a high frequency of spontaneous induction occurred, indicating that although phage ϕ42 and phage PS42-D are similar, they are probably not identical.

All of the evidence reported here suggests that the mechanism of β-lysin conversion mediated by the triple-converting phages ϕ42, ϕA1 and ϕA3 and the double-converting phage ϕ13 involves insertional inactivation of the chromosomally located *hlb* determinant by the integration of circularly permuted phage genomes. The negative conversion of *S. aureus* lipase by phage L54a has been shown to occur by a similar mechanism (Lee & Iandolo, 1986*a*). Because the sizes of the small *Hin*dIII-generated junction fragments, detected in *Hin*dIII-cleaved genomic DNA of lysogens harbouring phages ϕ42, ϕA1, ϕA3 and ϕ13 with *hlb* and *attP* probes, were identical, it is likely that these phages integrated at the same site, or very closely linked sites, in the bacterial chromosome. Furthermore, because the sizes of the two junction fragments, detected with the 2·2 kb *hlb* probe in *Hin*dIII-cleaved genomic DNA of three

independently generated lysogens in the case of all four phages, were consistent, these data suggest that these phages integrate in one orientation only. Similar findings have been reported for the *S. aureus* phages L54a and ϕ11, both of which integrate into the *S. aureus* genome in one orientation (Lee & Iandolo, 1986*b*, 1988).

Expression of the cloned *sak* determinants by *E. coli* derivatives on fibrin agar was more readily detectable around colonies of strain DS410 than HB101 or C600. Similar findings have been observed with regard to the expression by *E. coli* of cloned *S. aureus hlb*, *entA* and δ-lysin determinants (D. C. Coleman, unpublished data). Previous studies revealed that recombinant staphylokinase and β-lysin expressed by *E. coli* were preferentially located in the periplasmic space and cytoplasm, respectively, but in both cases the recombinant proteins escaped into the surrounding medium, probably due to lysis of ageing cells (Sako *et al.*, 1983; Coleman *et al.*, 1986). Because of the intrinsic properties of the minicell-producing strain DS410, it may be more leaky than either strain HB101 or C600, or it is possible that DS410 cells lyse earlier in the growth cycle. However, these findings indicate that DS410 is an extremely useful strain for studying the expression in *E. coli* of cloned genes derived from Gram-positive bacteria.

The discovery of triple-converting phages suggests that they can play an important role in controlling the expression of *S. aureus* extracellular proteins. This, together with transfer of virulence factors between *S. aureus* strains, may contribute to the generation of strains with enhanced pathogenicity. The presence of triple-converting phages in Irish methicillin-resistant isolates parallels the findings of a recent study from this laboratory which indicated that the capacity to produce enterotoxins by these organisms was an important contributory factor in their pathogenic potential (Humphreys *et al.*, 1989).

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