Sau42I, a BcgI-like restriction–modification system encoded by the Staphylococcus aureus quadruple-converting phage \( \phi 42 \)

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The serotype F phage \( \phi 42 \) of Staphylococcus aureus is a triple-converting bacteriophage that encodes the staphylokinase gene (\( sak \)) and the enterotoxin A gene (\( entA \)). Lysogeny results in loss of expression of the chromosomal \( \beta \)-haemolysin gene (\( hlb \)) (negative conversion), the expression of staphylokinase and enterotoxin A (positive conversion), and the acquisition of resistance to lysis by all 23 phages of the International Basic Set (IBS) of S. aureus typing phages. Until this study, the basis of \( \phi 42 \) resistance to lysis by exogenous phages was unknown. The authors report here that phage \( \phi 42 \) encodes a restriction–modification (R–M) system, termed Sau42I, adjacent to and in the same orientation to the phage integrase gene \( \text{int} \). The genes encoding Sau42I were cloned and sequenced, and found to consist of two overlapping reading frames, ORF S (specificity) and ORF RM (restriction–modification), in the same orientation. The ORFs share a high degree of DNA and amino acid sequence homology with the previously characterized BcgI R–M system of Bacillus coagulans. Expression of the cloned Sau42I ORF S and ORF RM in S. aureus 80CR3 transformants from a plasmid vector conferred resistance to lysis by all 23 IBS phages. Similarly, transformants of S. aureus RN4220 harbouring recombinant plasmids containing both ORFs were resistant to lysis by the IBS typing phages. However, transformants harbouring plasmids encoding either ORF S or ORF RM were susceptible to lysis by the IBS phages, and they had the same phage-susceptibility pattern as the respective parental isolates. In vitro analysis of crude and partially purified extracts of S. aureus transformants harbouring both the \( \phi 42 \) ORF S and ORF RM genes indicated that Sau42I has endonuclease activity and requires co-factors \( \text{Mg}^{2+} \) and \( S\)-adenosylmethionine in order to function, and activity is optimized at pH 8, although the precise recognition sequence has yet to be determined. The findings of this study confirm that \( \phi 42 \) is a quadruple-converting phage, believed to be the first described for S. aureus, and show that it encodes a novel R–M system termed Sau42I.

INTRODUCTION

In bacteria, simultaneous insertional inactivation of a chromosomal gene accompanied by acquisition of additional determinants encoded by a bacteriophage can result following lysogenization. This phenomenon is known as lysogenic conversion, whereby lysogenized bacteria acquire or lose (i.e. are converted) the ability to express various phenotypic traits, including the ability to produce specific extracellular enzymes and toxins. This type of conversion is
classified as single-, double- or triple-conversion depending on the number of genes affected. *Staphylococcus aureus* phages L54A and φ1 are single-converting phages, negatively and positively converting geh and lyt, respectively (Lee & Iandolo, 1986a, b, 1988; Lee & Buranen, 1989; Borchardt et al., 1993). The *S. aureus* serotype F phages φ13 and φ42 are double- and triple-converting phages, respectively, both of which inactivate the *hbl* gene in the *S. aureus* chromosome, and encode sak, or sak and entA, respectively (Coleman et al., 1986, 1989, 1991). Studies from this laboratory showed that these two phages integrate into the *S. aureus* chromosomal *hbl* gene in a site- and orientation-specific way by the action of the integrase gene, int (Carroll et al., 1993, 1995). Insertion occurs via the chromosomal *attB* site and the phage *attP* site, which share a 14 bp core sequence in common (Coleman et al., 1991; Carroll et al., 1993, 1995). Further studies from this laboratory found that lysogeny by φ42 not only results in triple conversion of the host strain, but simultaneously causes phage non-typability in strains previously susceptible to all 23 typing phages of the International Base Set (IBS) (Coleman et al., 1989). Our interest in φ42-conferred non-typability is partly due to the reported global increase of *S. aureus* clinical isolates that are also phage non-typable (Andrasevic et al., 1995; Tenover et al., 1995; Rosney et al., 2003).

The specific mechanisms by which *S. aureus* manages to exclude exogenous bacteriophages, and thereby become non-typable, have not been investigated in detail. It is generally considered that all *S. aureus* phages adsorb to their host cell surface, but may be prevented from further activity after DNA has entered the cell (Parker, 1983; Novick, 1990). Restriction–modification (R–M) systems are the best-known mechanisms for defence by bacteria against invasion by foreign DNA. One of the advantages of R–M systems over mutations conferring phage resistance is that one R–M system can simultaneously protect against a variety of phages, thus giving the host an advantage over competing organisms. In general, bacterial R–M systems consist of a restriction endonuclease (responsible for recognition of, and cleavage within, a specific DNA sequence) and a DNA methyltransferase (which modifies unmethylated or hemimethylated DNA within the same sequence) (Wilson & Murray, 1991; Cheng, 1995; Malone et al., 1995; Timinskas et al., 1995). Restriction endonucleases usually require Mg²⁺ or other divalent cations in order to cleave DNA. Some also require, or are stimulated by, ATP or Sadenosylmethionine (AdoMet). Modification methyltransferases require AdoMet as a co-factor, which serves as the methyl donor (Wilson & Murray, 1991; Cheng, 1995; Malone et al., 1995; Timinskas et al., 1995). In some R–M systems, the restriction endonuclease can operate independently of the modification machinery, while other systems require both the restriction and modification components in order to function (Wilson & Murray, 1991).

On the basis of a number of criteria, including enzyme subunit composition, cofactor requirements, DNA specificity and reaction products, R–M systems have been separated into three classes designated I, II and III (Yuan, 1981). However, new systems are being discovered that do not fit into this original classification. A rapidly growing new class of R–M systems includes the Bgl-like enzymes (Kong et al., 1993, 1994; Kong & Smith, 1997; Kong, 1998), which to date includes BaeI (Sears et al., 1996), BplI (Vitkute et al., 1997), Cjel and CjePl (Vitor & Morgan, 1995), Bsp24I (Degtyarev et al., 1993), and finally the one reported in this present study, Satt42I, which is the only one other than Bgl to have its gene sequences characterized in detail. An unusual feature of this family is that the enzymes cleave on both sides of the recognition sequence. They have been classified as type IIb restriction endonucleases (Roberts et al., 2003).

We have found two overlapping unidirectional ORFs adjacent to the *int* gene region of the *S. aureus* phage φ42 that have extensive nucleotide and amino acid sequence homology to the R–M system Bgl from *Bacillus coagulans*. The purpose of this study was to clone, analyse and express these two ORFs in *S. aureus*, and to investigate if they encode a functional R–M system that is responsible for the phage non-typable phenotype expressed by *S. aureus* φ42 lysogens.

## METHODS

### Bacterial strains, bacteriophages, plasmids, growth conditions and DNA purification.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was routinely grown in L broth (LB, Oxoid) or on L agar (LA, Oxoid). *S. aureus* strains were grown in Trypticase Soy Broth (TSB, Oxoid) or on Trypticase Soy Agar (TSA, Oxoid). Antibiotics were purchased from Sigma-Aldrich, and were used at the following concentrations for *E. coli* (*S. aureus* in parentheses): ampicillin 10 μg ml⁻¹, tetracycline 8 μg ml⁻¹ (3 μg ml⁻¹), chloramphenicol 30 μg ml⁻¹ (10 μg ml⁻¹). Phage φ42 was originally isolated from *S. aureus* strain PS42D, which is the propagating strain for the typing phage φ42D (Coleman et al., 1989). In this study, φ42 was propagated on the phage-free *S. aureus* host strain 80CR3 (Stoberingh & Winkler, 1977). *S. aureus* transformations were carried out with *S. aureus* strains 80CR3 and RN4220 (Table 1). Plasmid DNA preparations, agarose gel electrophoresis, and other routine molecular biology methods, were carried out as described by Sambrook et al. (1989).

### Phage typing of isolates.

The IBS of typing phages was supplied by the Central Public Health Laboratory, Colindale, UK, and phage typing of isolates was carried out as described by Blair & Williams (1961).

### Bacteriophage DNA preparation and purification.

To obtain high concentrations of phage φ42 particles for DNA analysis and cloning, *S. aureus* 80CR3 was grown for 12–16 h in 5 ml phage broth, according to Blair & Williams (1961), containing 0-01 M CaCl₂ at room temperature, and then transferred to an orbital shaker at 150 r.p.m. for 2 h at 37 °C. Bacteriophage particles were added to 1 ml cells at a ratio of approximately 1:10 in a sterile glass 5 ml Kahn tube, and incubation continued for 20 min for phage adsorption at room temperature. The mixture of phage and cells was then transferred to a 2 l Erlenmeyer flask containing 200 ml phage broth with 0-01 M CaCl₂, and incubated in an orbital shaker at 150 r.p.m. at 37 °C for 6–8 h, or until complete lysis had taken place. This was detected by observing a drop in the optical density of the culture, or the appearance of fine thread-like material and cellular debris resulting from cell lysis. Phage particles were collected, and their DNA was extracted as described below.
Phage φ42 DNA was purified from 500 ml lysis cultures using a modification of a method for purification of bacteriophage lambda DNA (Sambrook et al., 1989). The lysed cultures were treated with DNase (10 μg ml⁻¹) and RNase (25 μg ml⁻¹) for 2–4 h at room temperature. Sodium chloride (1 M final concentration) was then added to the lysate, dissolved by swirling, and placed on ice for 1 h. The culture was then centrifuged for 5 min at 6000 g. The supernatant was transferred to clean centrifuge buckets, and solid PEG MW 8000 was added to the lysate, dissolved by swirling, and placed on ice for 1 h. The solution was centrifuged at 6000 g for 10 min, and the supernatant was carefully decanted. The resulting pellet containing the phage particles plus PEG was then resuspended gently in 1–2 ml SM buffer (per litre: 5–8 g NaCl; 2 g MgSO₄·7H₂O; 50 ml 1 M Tris/HCl, pH 7.5; 5 ml 2 %, w/v, gelatin) using a sterile Pasteur pipette, and 0–5 ml was transferred to a sterile Eppendorf tube. The solution was then extracted two or three times with an equal volume of liquid phenol equilibrated with 10 mM Tris/HCl buffer, pH 8, and once with a phenol/chloroform mixture (1:1, v/v) before the DNA was finally precipitated with 2 vols absolute ethanol. The DNA was recovered by centrifugation, and washed once with 70 % (v/v) ethanol, dried briefly and resuspended mixture (1 : 1, v/v) before the DNA was finally precipitated with 2 vols absolute ethanol. The DNA was recovered by centrifugation, and washed once with 70 % (v/v) ethanol, dried briefly and resuspended with 100 ml lysis buffer, and 500 U lysostaphin was added. The cells were incubated for 20 min at 37 °C on a shaking incubator. After 20 × 1 min pulses on a Branson Sonifier (equipped with a 5 mm tip) at 4 °C, the cell debris was removed by centrifugation as described above, and the resulting supernatant was immediately poured onto a 15 × 5 cm heparin sulphate column (Pharmacia LKB). The active fractions from this step were eluted with a 200 ml linear gradient of 0-05–1-0 M NaCl in buffer A (see above). To detect the active fractions (i.e. those with DNA cleavage activity), 5 μl of each fraction was incubated with phage λ DNA for 5 min at 37 °C, and samples were tested by gel electrophoresis. Maximum activity was observed in the samples obtained at approximately 700 mM NaCl. Fractions showing some activity were pooled, and then run on a 15 × 2-5 mM source Q column (Pharmacia LKB). The active fractions from this step were detected as described above, pooled, and dialysed against 50 % (w/v) glycerol in buffer for 16 h at 4 °C to remove salts.

**Western immunoblotting.** Polyclonal antibodies raised against the 
Bcgl large and small subunits (Kong, 1998) were tested against cell extracts of *S. aureus* transformants as follows. Crude cell extracts and column-purified fractions of transformed and untransformed *S. aureus* were electrophoresed on SDS-PAGE gels, and transferred to a nitrocellulose filter by electroblotting. The nitrocellulose filter was then incubated for 1 h with rabbit primary antibody raised against Bcg. The filter was then washed before the addition of anti-rabbit antiserum linked to alkaline phosphatase (New England Biolabs) (Kong, 1998). The filter was then developed with the addition of 2–3 ml alkaline phosphatase substrate (NBT/BCIP; New England Biolabs), and the protein bands were viewed.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/phenotype*</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F’ [proAB+ lacI lacZΔM15 Tn10 (Tc’)]</td>
<td>Bullock et al. (1987)</td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80CR3</td>
<td>Hlb+ Sak– EntA–</td>
<td>Stobberingh &amp; Winkler (1977)</td>
</tr>
<tr>
<td>RN4220</td>
<td>Hlb+ Sak– EntA–</td>
<td>Kreiswirth et al. (1983)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
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<tr>
<td>pUC18</td>
<td>Ap⁺ lacZ</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pBluescript II KS (+/−)</td>
<td>lacZ</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pShCm</td>
<td>Ap⁺ lacZ Cm⁺ (E. coli–<em>S. aureus</em> shuttle plasmid)</td>
<td>Carroll et al. (1995)</td>
</tr>
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*Hlb, beta haemolysin; Sak, staphylokinase; EntA, enterotoxin A.

**Table 1. Bacterial strains and plasmids**
Transformation of bacteria. Competent E. coli cells for plasmid transformation were prepared, and transformed according to the method outlined by Sambrook et al. (1989). S. aureus electroporation-competent cells were prepared as follows. A 1 ml aliquot of cells from an overnight culture was inoculated into 100 ml TSB, and grown for a further 1-5 h to an OD$_{600}$ of 0.5. The cells were harvested at 6000 g for 10 min, and the cells were resuspended in 50 ml ice-cold (0-5 M) sucrose solution. The cells were harvested as before, resuspended in 1 ml sucrose solution, and transferred to a sterile 1-5 ml Eppendorf tube on ice for 20 min. Following incubation, the cells were harvested by centrifugation, and resuspended in 1 ml 0-5 M ice-cold sucrose solution. Aliquots of competent cells (200 ml) were transferred to 0-5 mm electroporation cuvettes (Bio-Rad) with 0.1-10 µg DNA, and electroporated at the following settings: 2-5 mV and 100 Ω. Immediately after electroporation, 500 µl TSB was added to the cells, and they were incubated at 37°C for 1 h before being plated on appropriate selective agar medium. Transformant colonies were usually observed after 24-48 h.

Construction of recombinant plasmids, and DNA sequencing. Plasmid pRD-RM10 (Fig. 1c), which encodes the complete putative S. aureus φ42 R–M system, was constructed by digesting pDC107 (Carroll et al., 1995) with NsiI–PstI and ligating the resulting 1-8 kb fragment containing sequences with homology to the specificity subunit (S) of Bcg to PstI-digested pRD21. Plasmid pRD21 (Fig. 1c) was constructed as follows. A 6-5 kb PstI–SalI fragment from φ42 DNA (Fig. 1b; Coleman et al., 1989), which contains some homology to the restriction methylation RM gene of Bcg, was cloned into the PstI–SalI site of pBluescript KS to yield plasmid pRDSK20. This plasmid was digested with ClaI, which produced two fragments: a 6-5 kb fragment, which contained the plasmid vector and the region of interest, i.e. the endonuclease modification gene; and a fragment of 2-5 kb containing sequences upstream from the remaining portion of the PstI–SalI fragment of φ42. This digested plasmid DNA was then religated, and transformed into E. coli. Plasmid pRD21 was selected from the transformants for further study.

For plasmid amplification in E. coli, and gene expression in S. aureus, DNA fragments from either pDC107 or pRD-RM10 were subcloned into the shuttle vector pSHCm (Carroll et al., 1995), which contains E. coli origins of replication, an ampicillin-resistance determinant from pUC19, replication functions from S. aureus plasmid pCW59, and a chloramphenicol-resistance determinant that is expressed in S. aureus (Wilson et al., 1981). The shuttle plasmid derivatives constructed included pRD21Cm1, pRD21Cm2, pRD21Cm3, and pRD21Cm4 (Fig. 1d). DNA for sequencing purposes was initially mapped using restriction endonucleases, and then subcloned to yield smaller inserts. Alternatively, deletion derivatives were constructed using the Erase-a-base kit (Promega). For sequencing the resulting subclones, the universal and reverse sequencing primers suitable for pUC18 and pBluescript KS+ were used, unless otherwise stated.

Bioinformatics. Database searches and sequence comparison were performed using the CLUSTAL W and BLAST algorithms (Higgins & Sharp, 1988; Altschul et al., 1990), and the National Center for Biotechnology Information (NCBI) GenBank/EMBL databases.

RESULTS

Localization and cloning of a putative R–M system from φ42

We have previously reported that S. aureus strains that are susceptible to infection by the IBS typing phages become immune to lysis by exogenous phages when lysogenized by phage φ42 (Coleman et al., 1989). This led us to believe that an efficient mechanism for preventing lysis by exogenous phage in φ42 lysogens was encoded within the φ42 genome. A clue to the location of the determinant(s) responsible was provided by our previous studies on the mechanism of integration of φ42 into the S. aureus genome (Carroll et al., 1995). From sequence data obtained from earlier studies on the int gene of φ42, an unassigned ORF, termed ORF A, was found upstream from int in the same orientation and in the direction of the attP site (Carroll et al., 1995) (Fig. 1c). ORF A and its predicted translation product were subsequently found to have significant DNA and amino acid sequence homology to subunit S, the putative specificity subunit of BcgI, a R–M system encoded by B. coagulans (Kong et al., 1994). The BcgI R–M system has features of both type I and III R–M systems. It is a heterooligomeric enzyme consisting of a small subunit, which contains the recognition or specificity function (S), and a larger subunit, which acts as a restriction endonuclease and methyltransferase (RM). The enzyme is unusual in that it cuts double-stranded DNA at both sides of the recognition site. The coding sequences for the two subunits are in overlapping reading frames on the B. coagulans genome (Kong et al., 1994; GenBank/EMBL accession no. L17341). From these similarities, we predicted that phage φ42 encodes a similar R–M system, and therefore further cloning and sequencing was undertaken. We also speculated that the putative φ42-encoded R–M system may be responsible for the broad-spectrum resistance to phage lysis observed previously in lysogens of phage φ42, and in this study we set out to investigate this possibility.

Subcloning and sequencing φ42 R–M genes

Fig. 1(a, b) shows the structural organization of the linear φ42 genome. ORF A, now renamed ORF S (see below), is located within a 5 kb HindIII fragment, and was originally cloned in plasmid pDC107 (Fig. 1c; Carroll et al., 1995). This plasmid was found to also contain a partial ORF with sequence homology to the 3’ end of the BcgI RM subunit. S. aureus ORF A was therefore renamed ORF S (specificity subunit) in analogy with the BcgI system, and the partial ORF was designated ORF RM (restriction and methyltransferase subunit) (Fig. 1c). The adjoining fragment on the phage genome was cloned from purified φ42 DNA on a 4 kb PstI–Clal fragment (Fig. 1b) into vector plasmid pBluescript KII KS+ - plasmids were used, unless otherwise stated.

Bioinformatics. Database searches and sequence comparison were performed using the CLUSTAL W and BLAST algorithms (Higgins & Sharp, 1988; Altschul et al., 1990), and the National Center for Biotechnology Information (NCBI) GenBank/EMBL databases.

Sau42I, a putative novel R–M system

Based on the findings described above, we believe that we have discovered a new R–M system encoded by S. aureus
Fig. 1. Schematic diagram of φ42 DNA showing the relative positions of genes involved in quadruple conversion. (a) Restriction endonuclease cleavage site map of the linear φ42 genome (taken from Coleman et al., 1989). (b) An enlarged section showing the relative positions of the entA and sak genes. (c) Phage φ42 DNA segments cloned in various plasmids used in the present study. Plasmid pDC107 (Coleman et al., 1989; Carroll et al., 1995; this study) showing the positions of the phage attachment site (attP), the integrase gene (int), ORF S, encoding the specificity gene of the R–M system, and a truncated ORF RM, encoding the R–M gene. Plasmid pRD21 encodes a truncated ORF RM gene, whereas pRD-RM10 encodes intact ORF S and ORF RM genes. All three plasmids can only replicate in E. coli. Plasmids pRDCm11, pRDCmT1, pRDCmT2 and pRDCmT3 are derivatives of shuttle plasmid pShCm (denoted by dashed lines either side of the cloned fragment) that can replicate in E. coli and S. aureus. The direction of transcription of the various genes is indicated by arrows. (d) S. aureus 80CR3 and RN4220 transformant derivatives harbouring pRDCm11 or pRDCmT1 were resistant to lysis by all 23 IBS typing phages. Plasmid pRDcT1 encodes a truncated ORF S lacking 20 aa from the C-terminal end of the protein, and a complete copy of ORF RM. Transformant derivatives harbouring pRDcT2, encoding a truncated ORF S lacking 174 aa from the C-terminal end (approx. half the protein), or pRDcT3, encoding an intact ORF S and a severely truncated ORF RM encoding 112 C-terminal aa only, or the shuttle vector plasmid pShCm only, were susceptible to lysis by all 23 IBS phages. Abbreviations: Av, AvaI; C, ClaI; H, HindIII; E, EcoRI; P, PstI; M, MboI; N, NsiI; Nu, NruI; RV, EcoRV; S, SalI.
bacteriophage ϕ42, and we have therefore named this putative system Sau42I.

The genetic organization of Sau42I is similar to that of the BcgI R–M system, i.e. the genes are encoded by two ORFs of the same orientation, but in different reading frames which overlap by several bases (Fig. 1c). The G+C content of the overall sequence is 32 mol%, which is similar to the content of other S. aureus phage genes, which averages 30 mol%; the G+C content of S. aureus bacterial genes averages 35 mol% (Novick, 1990).

The DNA sequence of ORF S, the putative specificity subunit, contains two contiguous potential ATG start codons in the same reading frame. Either of two translation products of 337 or 336 aa residues could therefore be produced with a similar molecular mass of approximately 38 kDa. Analysis of the amino acid sequence revealed that the second half of the protein sequence shows similarity to the first half, suggesting dimer symmetry in the protein. The amino acid homology between ORF S and the BcgI specificity subunit is 36 % identity and 50 % similarity. This is significantly lower than the level of homology between the RM subunits. An amino acid alignment of Sau42I specificity subunit ORF S and the BcgI S subunit is shown in Fig. 2. A search of the GenBank database with the predicted protein sequence indicated the closest homology to BcgI, and partial homology to a putative type IIIS restriction enzyme from the Helicobacter pylori genome (accession nos AE001559 and AE000647, respectively).

The DNA sequence of ORF RM, the putative restriction and modification subunit, shares 60 % identity with the RM subunit of BcgI. The deduced proteins encoded by Sau42I ORF RM and the BcgI RM subunit (subunit A) have 56±5 % amino acid identity, and 72 % residue similarity. An amino acid alignment between the two proteins is shown in Fig. 3. Three potential ATG start codons are present in the same reading frame of ORF RM. Depending on which of these is used, translation of ORF RM could yield a protein of between 627 and 639 residues, with an approximate molecular mass of between 68 and 70 kDa.

Expression of cloned Sau42I R–M genes in S. aureus confers resistance to lysis by exogenous phages

The evidence from the nucleotide and amino acid sequence data described above suggests that Sau42I belongs to the class of R–M enzymes similar to BcgI (Kong et al., 1994). In order to examine if Sau42I ORF RM and ORF S constitute an active R–M system in S. aureus, various fragments of pRD-RM10 encoding ORF S and ORF RM (Fig. 1c) were subcloned into the shuttle plasmid pShCm, which is capable of replicating in both E. coli and S. aureus (Carroll et al., 1995). The recombinant shuttle plasmid derivatives were then transformed into S. aureus strains 80CR3 and RN4220, and tested for susceptibility to the IBS typing phages. S. aureus 80CR3 was susceptible to lysis by all 23 phages of the IBS S. aureus typing phages, whereas RN4220 was susceptible to 17 of the IBS phages. Transformant derivatives of both strains, harbouring pRDcm11 encoding ORF S and ORF RM (Fig. 1c, Fig. 4), were resistant to lysis by all 23 IBS typing phages. Similar results were obtained with transformants harbouring pRDcmT1, which encoded ORF RM and a truncated ORF S lacking the C-terminal 20 aa residues (Fig. 1c). In contrast, transformants harbouring pRDcmT2, which encoded ORF RM and a truncated ORF S lacking the C-terminal 174 aa, had the same IBS phage-susceptibility pattern as the non-transformed parental strains. Similarly, transformants harbouring pRDcmT3, which encoded ORF S and a severely truncated ORF RM encoding the C-terminal 112 aa (Fig. 1c), and transformants harbouring the shuttle vector plasmid pShCm (Fig. 1c) only, had the same IBS phage-susceptibility pattern as the non-transformed parental strains. S. aureus transformants harbouring pRDcm11 or pRDcmT1 and wild-type ϕ42 lysogens always exhibited total resistance to lysis by IBS typing phages, and plaques

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**BcgI**

---MNLYKVSTTSLDLVYVIGK-----IDGNGAQRENENINTPIYTRKATNQRFGFPMID---

**Sau42I**

MNKLSAERKDFSLLEELFTVRKTTAPLILRKNKKERQKLPYTVTTSTYGIDPSFG

**BcgI**

-----EVEKLYGKLPLVRGHEES---KPVQETFPPFTGKVNIC1PKLDNRNHNCILVYTMNIA

**Sau42I**

-----PEEKNQTVADGDDYFQLWHFSASDREVEKLIPKLNKIVALFLVNCIA

**BcgI**

TKMDPS--YSYTMNSRLKSIIILDLLPIKGEFDYMNLYSIKLKSNMKNFVDQQQGDVS

**Sau42I**

TCEVQGYKPSQFQTRKQKGALPAGNQDFQFQFMERIKSKFNLRSIKQKQHEIND

**BcgI**

LRLKDSLWSQFKNDEIPS--IN6GVKLTADKMDKGPIFIIAGTSWNGTETSTSN--A

**Sau42I**

WREELIQETTSTQSTBTVNQIQAKITKVNQGFILTPY1565QINLQWDF1548KIR

**BcgI**

SFDWQILVGQVGYGVNYFAYPKVFSDDYKRKLKHKPNKVELLIMKTVLQORVKY

**Sau42I**

EFD--NCT1A1NSQVSGAFHTYFRIISAQDVT--KLKNKLHKFSFLYFIAMNRKLRLEQKY

**BcgI**

AVGYKFNATRMEQIILLTKADGDPYFENQYMQMNKQVGRTRKMEAD

**Sau42I**

SFNDRGINDFRERRILILLTK--RPYFENQYMQKNEEILLDRL---

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**Fig. 2.** Alignment of amino acid residues of the specificity subunits ORF S of BcgI and Sau42I. Proteins were aligned using CLUSTAL W (Higgins & Sharp, 1988). Perfectly conserved residues are indicated by an asterisk, conservative residue substitutions by two dots, and semi-conservative substitutions by one dot.
Fig. 3. Amino acid alignment of the Sau42I and BcgI RM subunits. The GenBank database accession numbers for the sequences are as follows: *S. aureus* Sau42I, X94423; *B. coagulans* BcgI, L17341. Sequences were aligned using CLUSTAL W (Higgins & Sharp, 1988). Perfectly conserved residues are indicated by an asterisk, conservative residue substitutions by two dots, and semi-conservative substitutions by one dot. Conserved motifs (CM) found in methyltransferases are shown in bold according to Timinskas et al. (1995). The CM I and CM II, and possibly the CM Is, motifs are thought to be involved in AdoMet binding and methyl-group transfer in class N methyltransferases (Timinskas et al., 1995). The DXXXGXG (where X is any amino acid) and NPPY submotifs present in CM I and CM II, respectively, are underlined.

Fig. 4. Susceptibility of *S. aureus* to lysis by IBS typing phages before and after transformation with *w*42-encoded Sau42I genes. (a) Phage non-typable phenotype exhibited by *S. aureus* RN4220 transformant harbouring plasmid pRDCm11 encoding ORF S and ORF RM. Similar phage non-typable phenotypes were exhibited by *S. aureus* 80CR3 transformants harbouring pRDCm11, and by *S. aureus* 42CR3-L, a *w*42 lysogenic derivative of 80CR3 (Table 1). (b) Phage-susceptibility profile exhibited by *S. aureus* RN4220 harbouring the shuttle vector plasmid pShCm. A similar phage-susceptibility profile was exhibited by RN4220. *S. aureus* 80CR3 is susceptible to lysis by all 23 IBS phages, whereas RN4220 is susceptible to lysis by only 17 of the IBS phages. (c) Pattern of IBS typing phages used for phage-susceptibility testing.
were never obtained with either high-density phage stocks or dilutions. These findings show that ORF S and ORF RM encoded by ϕ42 are responsible for the phage non-typable phenotype exhibited by S. aureus derivatives lysogenized by ϕ42, and that both genes are required for this to occur.

**In vitro detection of Sau42I endonuclease activity**

In order to study the putative R–M system in detail, *in vitro* studies were carried out on extracts of *S. aureus* strain RN4220 transformed with pRDCm11. In the absence of a specific antibody to detect *Sau*42I, antibodies raised against *Bcg* were used in an attempt to detect reactive proteins in the *S. aureus* extract because of the observed similarities between the *Sau*42I and *Bcg* R–M systems at the amino acid level (Figs 2 and 3). Crude cell extracts were prepared as described in Methods. Western blots were carried out on SDS-PAGE gels containing extracts of *S. aureus* RN4220 and transformant derivatives harbouring pRDCm11 encoding both ORF S and ORF RM. Purified *Bcg* was used as a positive control. The anti-*Bcg* antibodies detected both the 70 kDa RM component and the 39 kDa S component of *Bcg* (Fig. 5). A band at approximately 70 kDa was detected in the *S. aureus* transformant extracts that was similar in size to the *Bcg* RM component (Fig. 5). However, no 39 kDa band corresponding to the S component was detected (Fig. 5). A non-specific intermediate-sized band of approximately 55 kDa was detected in extracts of *S. aureus* RN4220 and *E. coli* DH5α, and in transformant derivatives of both strains harbouring pRDCm11 or the shuttle vector plasmid pShCm.

Endonuclease activity was detected on purified λ DNA with crude and partially purified cell extracts of *S. aureus* RN4220 transformants harbouring pRDCm11, but not with extracts from the plasmid-free parental strain. Large-scale extractions and purifications were carried out on *S. aureus* RN4220 transformants harbouring pRDCm11 in an attempt to further investigate endonuclease activity, and to obtain complete digestion of substrate DNA. Antibody-reactive fractions were detected by Western blotting in order to conserve the maximum amount of activity. The relevant fractions were pooled, and tested for endonuclease activity. Assays were carried out on a number of different DNA substrates, including purified λ DNA, pUC19 DNA and *S. aureus* genomic DNA. Partial digestion of the substrate DNA was observed; however, despite repeated cycles of purification, complete digestion was not obtained with any of the DNA substrates, and no difference in levels of digestion was observed following 5 or 60 min incubation (data not shown). Even though only partial digestion of substrate DNA was achieved, empirical studies revealed that the endonuclease has an absolute requirement for Mg2+ and AdoMet, and digestion was optimal at 37 °C and pH 8 (data not shown).

**DISCUSSION**

Previous studies from this laboratory showed that the *S. aureus* serotype F triple-converting phage ϕ42 confers broad-range phage non-typability on lysogenized host strains. Lysogenic derivatives of *S. aureus* 80CR3 were found to be resistant to lysis by all 23 phages of the IBS of typing phages, whereas the non-lysogenized parental strain was susceptible to lysis by all 23 IBS phages (Coleman *et al.*, 1989). The purpose of the present study was to investigate the mechanism of ϕ42-mediated resistance to phage lysis. A clue to the possible mechanism of resistance to phage lysis was found during an earlier study from this laboratory on the molecular mechanism of ϕ42 DNA integration into the *S. aureus* chromosome (Carroll *et al.*, 1995). During this study, an unassigned ORF and an overlapping partial ORF were found immediately adjacent to, and in the same direction as, the phage integrase gene *int* (Fig. 1c). The complete ORF, termed ORF A, was found to share significant nucleotide sequence and amino acid homology with the specificity (S) subunit of the *B. coagulans*-encoded R–M system *Bcg* (Kong *et al.*, 1993, 1994). This system consists of two overlapping genes in the same orientation: the smaller encodes the S subunit responsible for target recognition, and the larger encodes the R–M (RM) subunit encoding endonuclease and methyltransferase activity (Kong, 1998). The ϕ42 partial ORF shared significant nucleotide sequence and amino acid homology with the RM gene of *Bcg*. These findings suggested that ϕ42 encodes a R–M system similar to *Bcg*, and that this may be responsible for immunity to lysis by exogenous phages exhibited by ϕ42 lysogens. In order to investigate this possibility, the ϕ42 DNA region upstream from the partial ORF was cloned, and the complete nucleotide sequence of the ORF was

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**Fig. 5.** Western blot of *S. aureus* and *E. coli* cell extracts with antibody raised against the S and RM subunits of *Bcg*. Lanes: 1, *S. aureus* RN4220; 2 and 3, RN4220 transformants harbouring pRDCm11 (encoding ORF S and ORF RM); 4, *E. coli* DH5α transformant harbouring pRDCm11; 5, purified *Bcg*. The antibody was found to cross-react with an unknown, intermediate-sized band (approx. 55 kDa) in both transformed and untransformed *E. coli* and *S. aureus* cell extracts.
determined. This ORF was named ORF RM in analogy with the \textit{Bcg} R–M system (Fig. 1c). The $\phi 42$ ORF A was renamed ORF S in analogy with the \textit{Bcg} R–M system. The predicted proteins encoded by the \textit{Bcg} and $\phi 42$ ORF S and ORF RM genes shared significant homology at the amino acid level: 36\% residue identity and 50\% similarity for the S subunits, and 56:5\% identity and 72\% similarity for the RM subunits, respectively. These findings were reflected by the results of Western blotting experiments with polyclonal antiserum raised against \textit{Bcg}, in which only the \textit{Sau}42I methyltransferase subunit reacted with the antiserum, whereas the S subunit with lower residue identity to \textit{Bcg} did not (Fig. 5). For both systems, the 3′ end of ORF RM overlaps the 5′ end of ORF S by several bases. Furthermore, in both systems, ORS S and ORF RM have different reading frames, and both have the same orientation (Fig. 1c). These findings further strengthened the suggestion that $\phi 42$ encodes a R–M system similar to \textit{Bcg}. This putative R–M system was named \textit{Sau}42I.

In order to investigate whether \textit{Sau}42I is functional in \textit{S. aureus}, a $\phi 42$ DNA fragment encoding both genes was cloned in shuttle plasmid pRDCm11 (Fig. 1c), and transformed into \textit{S. aureus} 80CR3. Transformants harbouring pRDCm11 were found to exhibit resistance to lysis by all 23 phages of the IBS typing set, as did \textit{S. aureus} 42CR3-L (Table 1), a $\phi 42$ lysogenic derivative of 80CR3. In contrast, 80CR3 was susceptible to lysis by all 23 IBS phages. Similarly, \textit{S. aureus} RN4220 transformants harbouring pRDCm11 were resistant to lysis by all the IBS phages, whereas the parental strain was susceptible to lysis by 17/23 IBS phages (Fig. 5). Interestingly, transformants harbouring pRDCmT1 (Fig. 1c), which encoded ORF RM and a truncated derivative of ORF S lacking sequences for the C-terminal 20 aa, were also resistant to lysis by all 23 IBS typing phages. Presumably the functional domains encoded by ORF S are still intact in plasmid pRDCmT1. Transformants harbouring pRDCmT2 encoding ORF RM and a truncated ORF S lacking the C-terminal 174 aa were susceptible to lysis by the IBS typing phages. Similarly, transformants harbouring pRDCmT3 encoding ORF S and a truncated ORF RM encoding the C-terminal 112 aa only were susceptible to lysis by the IBS typing phages. These findings demonstrated that the $\phi 42$ ORF S and ORF RM genes are responsible for the broad-range phage lysis resistance exhibited by $\phi 42$ lysogens, and that both genes are required for this to occur.

The observed homology between the ORF S and ORF RM determinants of the \textit{Sau}42I and \textit{Bcg} systems, and the resistance to lysis by exogenous phages of the IBS typing set exhibited by \textit{S. aureus} transformants harbouring cloned $\phi 42$ ORF S and ORF RM genes, strongly suggested that \textit{Sau}42I is a functional R–M system. Timinskas \textit{et al} (1995) classified the $\textit{Bcg}$-encoded methyltransferase protein as belonging to class N12, based on the presence and order in the primary sequence of characteristic conserved amino acid motifs. All methyltransferases identified to date contain conserved amino acid motifs, despite the fact that there is little overall sequence homology. Fig. 3 shows an amino acid alignment of the \textit{Sau}42I and \textit{Bcg} methyltransferase subunits, and the four conserved motifs (CM) identified by Timinskas \textit{et al} (1995) are present in both sequences. The submotif FXXGXG (where X can be any residue), located within CM I, is common to virtually all enzymes that utilize AdoMet for the source of methionine, apart from N12 methyltransferases such as \textit{Bcg} and \textit{Sau}42I, where instead of invariant F, other amino acid residues are found (Fig. 3). The submotif is believed to be the AdoMet binding domain (Wilson & Murray, 1991; Timinskas \textit{et al}, 1995).

The second motif present in both the \textit{Sau}42I and \textit{Bcg} amino acid sequences is CM II, comprising a sequence of 10 aa residues (Fig. 3). CM II motifs are present in all methyltransferases, and very often the sequence PPY is conserved (Timinskas \textit{et al}, 1995). The nature of the residues preceding PPY is characteristic for different classes of methyltransferase (D in D12 and D21 classes, S in S21 and S12, and N in N12), and it correlates with the base methylation specificity of the enzymes (Klimasauskas \textit{et al}, 1989; Timinskas \textit{et al}, 1995). This element appears to be unique to the amino methyltransferases (Zhang \textit{et al}, 1993). In the \textit{Sau}42I with \textit{Bcg} methyltransferase sequences the submotif NPPY is present within CM II, and thus both of these enzymes belong to class N12 (Fig. 3). Timinskas \textit{et al} (1995) reported that class N methyltransferases include a third CM, termed CM Is, preceding CM I. This sequence is usually 17 aa in length in almost all class N methyltransferases, and is present in both the \textit{Sau}42I and \textit{Bcg} (Fig. 3). In class N methyltransferases, CM I and CM II are thought to be involved in AdoMet binding and methyl group transfer (Timinskas \textit{et al}, 1995). Close location of CM Is to CM I in the primary amino acid sequence is indicative of possible interactions (Fig. 3; Timinskas \textit{et al}, 1995).

In order to investigate whether the ORF RM determinant encoded detectable endonuclease activity in \textit{S. aureus} transformants, crude and partially purified cell lysates of \textit{S. aureus} 80CR3 and RN4220 transformants harbouring plasmid pRDCm11 were investigated for endonuclease activity on bacterial, plasmid and phage DNA. Endonuclease activity was detected; however, despite repeated attempts, complete digestion of substrate DNA was never achieved. This was probably due to concurrent methylation and cleavage of substrate DNA by the endonuclease and methylation subunit encoded by ORF RM, resulting in a proportion of the substrate DNA being protected from cleavage. Thus the exact cleavage site of \textit{Sau}42I has not yet been elucidated. In the \textit{Bcg} R–M system, both the S and RM subunits are required to bind, cleave and methylate DNA (Kong \textit{et al},...
1994). Both the endonuclease and methylase domains are located in the RM subunit, with the target recognition domain located in the S subunit (Kong, 1998). Further studies are in progress to dissect the functional organization of the Sau42I system, and to identify the target recognition sequence.

The finding of an R–M system encoded by φ42 distinguishes this phage as a quadruple-converting phage, believed to be the first reported for \( S. aureus \). Lysogenization of \( S. aureus \) by φ42 or similar phages enhances the virulence potential of lysogens by giving them the ability to express enterotoxin A and staphylokinase. The ability of φ42 to confer protection on its host against lysis by a wide variety of exogenous phages, while not a virulence factor in itself, would confer a selective advantage over phage-susceptible cells in the presence of lytic phages, providing lysogens with a potentially significant survival advantage. It is possible that phages similar to φ42 may be partly responsible for the high prevalence of phage non-typable strains reported among clinical isolates of \( S. aureus \).

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**REFERENCES**


