The Fbe (SdrG) protein of *Staphylococcus epidermidis* HB promotes bacterial adherence to fibrinogen

Orla Hartford,† Louise O’Brien,† Karin Schofield,‡ Jerry Wells§ and Timothy J. Foster†

Author for correspondence: Timothy J. Foster. Tel: +353 1 6082014. Fax: +353 1 6799294. e-mail: tfoster@tcd.ie

*Staphylococcus epidermidis* strains HB and K28 express surface proteins called Fbe or SdrG, respectively, that have sequence similarity to the clumping factors ClfA and ClfB of *Staphylococcus aureus*. A mutation in the *fbe* gene of strain HB was isolated by directed plasmid integration using the broad-host-range temperature-sensitive plasmid pG*M*Host9 (pVE6155). An internal fragment of *fbe* was cloned into pG*M*Host9 and the chimaeric plasmid was mobilized from *S.* *aureus* RN4220 to *S. epidermidis* 9142 by conjugation promoted by plasmid pGO1. The plasmid was then transferred to *S. epidermidis* strain HB by phage-48-mediated transduction. The plasmid integrated into the chromosomal *fbe* gene at a frequency of 2 × 10⁻⁴. All the survivors tested had a copy of pG*M*Host9‘fbe’ integrated into the chromosomal *fbe* gene either as a single copy or as a tandem array. Western immunoblotting showed that the wall-associated Fbe protein was absent in the mutant. Wild-type *S. epidermidis* HB adhered to immobilized fibrinogen in a dose-dependent and saturable fashion whereas the mutant did not bind. The Fbe proteins of HB and K28 were expressed at a high level in *Lactococcus lactis* MG1363 using the expression vector pKS80. These strains adhered strongly to immobilized fibrinogen. These results confirm that Fbe is a fibrinogen-binding adhesin.

**Keywords:** surface protein, *Lactococcus lactis*, adhesion, MSCRAMM

**INTRODUCTION**

*Staphylococcus epidermidis* is a common commensal of human skin. In the past 20 years this organism has emerged as a frequent cause of nosocomial infections associated with indwelling medical devices such as intravenous catheters, artificial heart valves and orthopaedic implants (Crossley & Archer, 1997). This is due to the ability of the organism to colonize the surface of the implanted material and to form a biofilm (Mack, 1999). Direct attachment to surfaces prior to implantation is considered to be a major route of infection. It has been suggested that expression of a polysaccharide adhesin (PS/A) is sufficient to promote attachment and subsequent biofilm formation (Tojo et al., 1988), while others have indicated that initial adhesion is mediated by a surface protein with polysaccharide being required only for the accumulation phase of biofilm formation (Heilmann et al., 1996, 1997; Mack et al., 1994).

Shortly after a plastic or metal device is introduced into the body, the surface of the device becomes coated with a conditioning layer of host proteins (Vaudaux et al., 1994, 1995; Francois et al., 2000). Foreign-body infection caused by *Staphylococcus aureus* is attributed to its ability to attach to host proteins such as fibronectin and fibrinogen that are in the coating layer (Vaudaux et al., 1989, 1994). Adherence is mediated by surface protein adhesins of the MSCRAMM (microbial surface component recognizing adhesive matrix molecule) family (Patti et al., 1994; Foster & Höök, 1998). The fibrinogen-binding proteins ClfA and ClfB have been shown to be important MSCRAMMs in foreign-body infections.
caused by *S. aureus* (Ni Eidhin et al., 1998; McDevitt et al., 1994).

Some strains of *S. epidermidis* can attach to fibrinogen immobilized on a plastic surface in vitro (Baldassarri et al., 1997; Herrmann et al., 1988; Nilsson et al., 1998) so it is possible that this organism can also initiate device-related infections by attaching to the conditioned surface of an implant within the body. A fibrinogen-binding protein, Fbe, has been identified in *S. epidermidis* strain HB (Nilsson et al., 1998; Pei et al., 1999). *S. epidermidis* K28 expresses a closely related protein which we called SdrG (McCrea et al., 2000). Fbe and SdrG are members of the Clf–Sdr multigene family of staphylococcal surface proteins (Fig. 1; Josefsson et al., 1998). They have a 50-residue signal sequence, a 548-residue characteristic of the Clf–Sdr family. The SdrG protein from strain K28 has 36 residues in the dipeptide Ser-Asp which are characteristic of the Clf–Sdr family. The SdrG protein from strain K28 has 216 residues. At the C-terminus the proteins have an LPXTG motif, a hydrophobic domain and positively charged residues associated with anchoring the protein to the cell surface (Navarre & Schneewind, 1999).

The recombinant A domain of Fbe from strain HB inhibited attachment of *S. epidermidis* strain 19 to immobilized fibrinogen in a concentration-dependent fashion (Pei et al., 1999), suggesting that Fbe is a fibrinogen-binding MSCRAMM. In this study we isolated a site-specific mutation in the *fbe* gene of *S. epidermidis* HB and expressed the Fbe and SdrG proteins from strains HB and K28 on the surface of the non-fibrinogen-binding organism *Lactococcus lactis*. Our data show that these proteins do promote bacterial adherence to fibrinogen and we now refer to SdrG as Fbe.

**METHODS**

**Bacterial strains and plasmids.** The *S. epidermidis* strains used in this study were K28 (McCrea et al., 2000), HB (Nilsson et al., 1998) and 9142 (Mack et al., 1994). Strain 9142 is resistant to norfloxacin and was used as a recipient in filter matings with *S. aureus* RN4220 carrying the conjugal gentamicin-resistance plasmid pGO1 (Archer & Johnston, 1983). *Escherichia coli* T61 is RecA⁺ and expresses the wild-type Rep protein of plasmid pWV01, which allows pG’Host9 derivatives to be stably propagated at 37 °C (Maguin et al., 1996). The 3752 bp broad-host-range temperature-sensitive shuttle plasmid pG’Host9 (Maguin et al., 1996) was used for gene inactivation experiments. Plasmid pG’Host9 (or pVE6155) is a variant of pG’Host4 (or pVE6004) (Maguin et al., 1992), created by ligating the SacI fragment from pG’Host4 carrying the *rep* gene and multiple cloning site with a BamHI fragment from pIL253 specifying erythromycin resistance which is selectable in both *E. coli* and Gram-positive hosts.

*L. lactis* strain MG1363 (Wells et al., 1993) was used to express Fbe proteins from the expression vector pKS80, a derivative of pTREX1 (Wells & Schoefeld, 1996) which provides a strong lactococcal promoter and a translational coupling mechanism to ensure efficient transcription and translation of genes cloned into the BcII site (Fig. 2).

**DNA manipulation.** DNA manipulations were performed using standard procedures. Plasmid DNA was isolated from *L. lactis* using the Wizard Plus SV miniprep kit (Promega) with the following modifications to the protocol supplied by the manufacturer. *L. lactis* cells were grown to late-exponential phase in 5 ml M17 broth. Cells were harvested and placed in resuspension buffer containing 500 U mutanolysin ml⁻¹ (Sigma) and 200 µg lysozyme ml⁻¹ (Sigma) and incubated at 37 °C for 30 min before addition of lysis buffer. Chromosomal DNA was isolated from *S. epidermidis* strains grown to stationary phase in BHI using the genomic DNA purification kit (Edge BioSystems) with 50 µg lysostaphin ml⁻¹ (AMBI, New York) incorporated into the spheroplast buffer and incubated at 37 °C for 10 min.

**Construction of pG’Host9’fbe’.** An 855 bp fragment was amplified from within the region of *fbe* (sdrG) encoding the unique A domain of the Fbe (SdrG) protein from *S. epidermidis* K28. The primer F1-GGCGAATTCCAAAGACCTCTACTGTCG annealed at position 435–454 and included an EcoRI site at the 5’ end (in italics) and primer R1-GGAATTCTTGGTATGCTTTCG annealed at position 1262–1280 and included a HindIII site at the 3’ end (in italics). The product was purified using Wizard PCR Prep (Promega), cut for 16 h with 20 units EcoRI and HindIII and ligated with pG’Host9 cut with the same enzymes. The ligated DNA was transformed into *E. coli* T61 and transformants were selected for by growth on erythromycin (100 µg ml⁻¹). DNA was isolated and transformed by electroporation (Oskouian & Stewart, 1990) into *S. aureus* RN4220(pGO1) selecting on gentamicin (10 µg ml⁻¹) and erythromycin (100 µg ml⁻¹).

**Transfer of pG’Host9’fbe’ into *S. epidermidis*.** Stationary-phase cultures of *S. aureus* RN4220(pGO1, pG’Host9’fbe’) and *S. epidermidis* 9142 grown in BHI broth were lawned on the surface of BHI agar and grown for 16 h at 30 °C. The lawns were emulsified in PBS and resuspended at an OD₆₀₀ of 10. Portions of 1 ml of each were mixed, centrifuged and resuspended in 100 µl PBS and pipetted onto a 2 cm² Protran nitrocellulose membrane (Schleicher & Schuell) on the surface of a BHI agar plate and incubated for 24 h at 30 °C. Cells were harvested in PBS and spread onto BHI agar containing 5 µg norfloxacin ml⁻¹ and 10 µg erythromycin ml⁻¹ and incubated
for 3–4 d at 30 °C. Transconjugants were screened for those that were sensitive to gentamicin. Phage 48 (Nedelmann et al., 1998) was propagated on one such transconjugant and pG’Host9 fbe’ was transduced into strain HB selecting for resistance to erythromycin at 30 °C.

Isolation of fbe mutants. S. epidermidis carrying pG’Host9 fbe’ was grown in TSB containing erythromycin (100 µg ml⁻¹) at 28 °C. Cultures were diluted and spread on TSA containing erythromycin and incubated at 43 °C.

Cloning of fbe in L. lactis. The fbe genes of S. epidermidis strains HB and K28 were amplified by PCR using Pfui polymerase from the putative translational initiation codon to the TAA stop codon and cloned into the L. lactis expression vector pKS80, which provides a lactococcal promoter, ribosome-binding sites and a translational coupling mechanism to ensure efficient initiation of translation of the cloned gene (Fig. 2). The primers used were LsdrGR1-CGCGGATCCTTAATTTTATTTTTCTAT-TTTTGGCAC. Both primers introduce a BglII site (italicized) to facilitate cloning of the product into the BglII site of the vector. The ligation mixture was transformed into L. lactis MG1363 cells made competent by washing and suspension in 0.5 M sucrose and 10% glycerol as previously described (Wells et al., 1993). The cells had been grown for 6 h in 300 ml M17 broth (Difco) containing 0.5% glucose at 30 °C without shaking. Transformants were selected on M17 agar incorporating 0.5% glucose and erythromycin (5 µg ml⁻¹) at 30 °C. Transformants expressing Fbe were identified by whole-cell immunoblotting. Cultures were grown to stationary phase in 2 ml M17 broth with 0.5% glucose and 5 µg erythromycin ml⁻¹ and 10 µl was spotted onto a Protran nitrocellulose membrane. After blocking with 10% skimmed milk for 1 h, membranes were incubated with anti-Fbe (SdrG) region A antibodies (1:1000; McCrea et al., 2000), washed three times with TBS, incubated with protein A coupled to horseradish peroxidase (1:500; Sigma) and detected by LumiGLO chemiluminescence substrate (New England Biolabs).

Detection of Fbe expression by Western immunoblotting. L. lactis transformants were grown to late-exponential phase in M17 broth containing 5 µg erythromycin ml⁻¹, washed twice in 5 mM EDTA, pH 8.0, and concentrated to an OD₆₀₀ of 40 in 1 ml 20% raffinose (Sigma) in 20 mM Tris/HCl, pH 8.0, 10 mM MgCl₂. Cell-wall-associated proteins were released by incubation at 37 °C with occasional shaking with 500 U mutanolysin ml⁻¹ and 200 µg lysozyme ml⁻¹ in the presence of protease inhibitors (Complete Mini; Roche) and 10 mM PMSF. Proteolasts were removed by centrifugation at 12000 g for 10 min and 200 µl of the supernatant was added to the final sample (Laemmli, 1970), boiled for 10 min and subjected to PAGE (4.5% acrylamide stacking gel, 7.5% acrylamide separating gel). Gels were electroblotted onto membranes as described previously (McCrea et al., 2000) and were incubated with antibodies and developed as described above.

S. epidermidis was grown for 3–5 h in 50 ml TSB, without shaking, at 37 °C to an OD₆₀₀ of 0.8. Cells were collected by centrifugation, washed in PBS, resuspended to an OD₆₀₀ of 10 in 250 µl raffinose buffer (see above) containing 80 µg lyso- staphin ml⁻¹ (AMBI, New York) and incubated for 20 min at 37 °C. Samples were prepared for electrophoresis and immunoblotting as described above.

Whole-cell dot immunoblot titrations used to compare the level of Fbe expression were performed as described by Hartford et al. (2001).

Southern hybridization. Genomic DNA (5 µg) was cleaved with 20 units HindIII and separated on a 0.8% agarose gel. Transfer of DNA to nitrocellulose membranes was performed as described previously (McCrea et al., 2000). The same PCR primers used in the construction of pG’Host9 fbe’ were used to amplify a fragment in the presence of DIG-dUTP (Roche). This was used as a probe in hybridization experiments. Hybridization was detected by chemiluminescence (Roche).

Adherence to immobilized fibrinogen. Adherence assays were performed in 96-well ELISA dishes as described previously (Hartford et al., 1997, 2001) with several modifications. L. lactis and S. epidermidis cells were grown under the same conditions used to prepare cells for releasing wall-associated proteins for Western immunoblotting. Cells were washed in PBS and resuspended to an OD₆₀₀ of 4 in PBS. Wells in ELISA dishes were coated for 16 h at 4 °C with human fibrinogen (Calbiochem; 0.01–20 µg ml⁻¹). Following blocking for 1 h at...
37 °C with 2 mg BSA ml⁻¹ (Sigma), wells were washed three times with PBS and 100 μl of a cell suspension was added and incubated for 2 h at 37 °C. Wells were washed three times with PBS and adherent cells were fixed by adding 100 μl 25 % formaldehyde and incubating at room temperature for 30 min. After washing three times with PBS, cells were stained with 5 % crystal violet for 1 min, washed three times with PBS and 100 μl 5 % acetic acid was added to each well to dissolve the cell-associated dye. The A₅₇₀ was measured in an ELISA plate reader.

RESULTS

Construction of pG⁺Host9’fbe’ and transfer into S. epidermidis

The broad-host-range temperature-sensitive plasmid pG⁺Host9 was used to generate a site-specific null mutation in the fbe gene of S. epidermidis HB. An 855 bp fragment from within the region that encodes the A domain of Fbe (SdrG) from S. epidermidis K28 was amplified by PCR and cloned into pG⁺Host9. Strain K28 was chosen because it was the organism from which the sdrG gene was first cloned (McCrea et al., 2000). The plasmid pG⁺Host9’fbe’ was established in E. coli strain T61 before being transformed into S. aureus RN4220(pGO1). Most strains of S. epidermidis, including strain K28, are not transformable with plasmid DNA, even at low frequencies. Conjugative plasmids from S. aureus are known to be transferable to S. epidermidis by filter mating and will promote transfer of small multicy copy plasmids (mobilization). Thus we mobilized pG⁺Host9’fbe’ from S. aureus by conjugation promoted by the gentamicin-resistance plasmid pGO1. S. aureus RN4220(pGO1, pG⁺Host9’fbe’ was filter-mated with S. epidermidis 9142, a strain that was known to be a suitable recipient for plasmid transfer by conjugation from S. aureus (Mack et al., 1994). Transconjugants that expressed erythromycin resistance were obtained at a low frequency (approx. 10⁻⁴ per donor). They were screened for sensitivity to gentamicin in order to identify those that did not inherit pGO1. We observed that the temperature-sensitive phenotype of pG⁺Host9 was suppressed in the presence of pGO1, in both S. aureus and S. epidermidis 9142. Thus gentamicin-sensitive pG⁺Host9 transconjugants that lacked pGO1 were identified. These occurred at a frequency of about 20%. Plasmid pG⁺Host9’fbe’ was then transduced by phage 48 to S. epidermidis strain HB. However, no transductants were obtained when K28 was used as a recipient.

Isolation and characterization of pG⁺Host9 insertions in the fbe gene of S. epidermidis HB

S. epidermidis HB bearing the ts plasmid pG⁺Host9’fbe’ was grown at 45 °C in order to isolate variants where the ts plasmid had integrated into the fbe gene to survive the restrictive temperature for plasmid replication. The efficiency of plating was 2·8 × 10⁻⁴. Colonies that grew were putative integrants and fbe mutants where the plasmid had integrated into and disrupted the chromosomal fbe gene. Several were picked and genomic DNA was analysed by Southern blot hybridization of HindIII-cleaved genomic DNA using a probe for the A region of fbe. The wild-type fbe (sdrG) gene is present on an approximately 12 kb fragment (Fig. 3; McCrea et al., 2000). The integrants fell into two classes: (i) those that lacked the wild-type fragment and possessed two smaller HindIII fragments of 10 kb and 6·2 kb that reacted with the probe (the additional HindIII site is contained within the plasmid vector); (ii) those that lacked the wild-type fragment and possessed three reactive fragments, two of which were the same as in the previous case and one that...
A high proportion of pG<sup>+</sup>D duplications was 1:8. The proportion of single-copy integration events to tandem event (McDevitt et al., 1993; Supersac et al., 1998). The proportion of single-copy integration events to tandem duplications was 1:8.

A high proportion of pG<sup>+</sup>Host9 integration events involved duplication. Also, the mutant Ts Rep protein of pG<sup>+</sup>Host9 is inactive at 37 °C, at least in lactococci (Biswas et al., 1993). It was deemed prudent to determine the stability of the two types of integrant after prolonged growth in the absence of selection at 37 °C. Thus two representatives of each were grown for 100 generations by serial dilution in drug-free broth at 37 °C. Cultures were diluted, plated on drug-free agar and approximately 500 colonies were replica-plated onto agar containing erythromycin. There was no detectable loss of erythromycin resistance. DNA was prepared from representative colonies and probed by Southern blotting of erythromycin resistance. DNA was prepared from approximately 500 colonies were replica-plated onto agar containing erythromycin. There was no detectable loss of erythromycin resistance. DNA was prepared from representative colonies and probed by Southern blotting of erythromycin resistance. DNA was prepared from approximately 500 colonies were replica-plated onto agar containing erythromycin. There was no detectable loss of erythromycin resistance. DNA was prepared from representative colonies and probed by Southern blotting of erythromycin resistance. DNA was prepared from approximately 500 colonies were replica-plated onto agar containing erythromycin. There was no detectable loss of erythromycin resistance. DNA was prepared from representative colonies and probed by Southern blotting of erythromycin resistance. DNA was prepared from approximately 500 colonies were replica-plated onto agar containing erythromycin. There was no detectable loss of erythromycin resistance. DNA was prepared from representative colonies and probed by Southern blotting of erythromycin resistance. DNA was prepared from approximately 500 colonies were replica-plated onto agar containing erythromycin. There was no detectable loss of erythromycin resistance. DNA was prepared from representative colonies and probed by Southern blotting of erythromycin resistance.

Expression of Fbe

Cell-wall-associated proteins were released from S. epidermidis strain HB and the fbe insertion mutant by digestion with lysostaphin in the presence of 30% raffinose. Western immunoblots were probed with anti-Fbe (SdrG) region A antibodies. A single immuno-reactive protein of 190 kDa was expressed by strain HB wild-type but not by the fbe mutant (Fig. 4).

To determine unambiguously if Fbe is a fibrinogen-binding adhesin, the fbe genes from strains HB and K28 were cloned into the L. lactis expression vector pKS80 and transformed into L. lactis strain MG1363. Preliminary analysis indicated that solubilizing cell-wall-associated proteins by incubation with mutanolysin and lysozyme for 60 min resulted in extensive breakdown of Fbe (Fig. 5 shows data for the protein from strain HB). When the incubation was shortened to 15 min, much less breakdown was observed and it was possible to detect an immunoreactive band the same size as the single band released from S. epidermidis HB. Quantitative whole-cell dot immunoblot experiments suggested that there was >500-fold more Fbe protein on the surface of L. lactis than the native S. epidermidis host (data not shown).

Adherence of bacteria to immobilized fibrinogen

Wild-type S. epidermidis HB, the fbe mutant and L. lactis expressing Fbe proteins from HB and K28 were tested for their ability to adhere to immobilized fibrinogen. Strain HB adhered in a dose-dependent and saturable fashion (Fig. 6a), whereas the fbe mutant was completely defective in binding. L. lactis cells expressing the Fbe protein from strain HB also adhered in a dose-dependent and saturable fashion (Fig. 6b). The number of cells that bound at saturation was sevenfold higher than for S. epidermidis HB. L. lactis expressing the Fbe protein from strain K28 gave similar results (data not shown). These data clearly show that the Fbe proteins of strains HB and K28 are fibrinogen-binding proteins capable of promoting bacterial adhesion to the immobilized ligand.
bacteria to immobilized fibrinogen substrates. They could thus contribute to the initiation of foreign-body infection by allowing bacteria to adhere to biomaterial surfaces that have become coated with host proteins after implantation. This mechanism could complement the previously described adherence of *S. epidermidis* to naked plastic surfaces, which presumably occurs prior to implantation.

Fbe was expressed at a sufficiently high level to allow detectable adherence only when *S. epidermidis* HB was grown to early exponential phase in broth without shaking. The failure of the fbe mutant of strain HB to adhere indicated that binding was due to the Fbe protein. However, even under optimum conditions adherence was poor compared to *L. lactis* expressing Fbe. We estimated that there was at least 500-fold more Fbe protein present on the surface of *L. lactis* compared to *S. epidermidis*. Enhanced expression by *L. lactis* was reflected by the higher level of bacterial adherence.

This paper thus demonstrates the merits of using a surrogate Gram-positive host to express a single protein to complement studies with null mutants of the natural host. *L. lactis* MG1363 has been used as a host for expression of large LPXTG-anchored surface proteins from *Streptococcus gordonii* (Holmes et al., 1998) and *S. aureus* (Que et al., 2000). We have used this system to express several surface proteins from *S. aureus* with equal success (L. O’Brien & T. J. Foster, unpublished data).

One problem that we have identified with *L. lactis* is that solubilization of the cell-wall-associated surface protein by mutanolysin and lysozyme treatment resulted in extensive degradation. We partially circumvented this by shortening the solubilization time from 60 min to 15 min. It is our belief that there is relatively little degradation of the protein on the surface of intact exponential phase *L. lactis* cells because of the high levels detected by quantitative whole-cell immunoblotting and because of the high dose-dependent and saturable adherence to fibrinogen. Proteases that are impervious to inhibition by the protease inhibitor cocktail must be activated during protoplast formation.

The apparent molecular mass of the Fbe protein of *S. epidermidis* HB (190 kDa) is higher than that observed for SdrG expressed by strain K28 (170 kDa; McCrea et al., 2000). This is most likely due to the K28 protein having an R region of 56 residues compared to that of the HB protein, which comprises 216 residues.

The pG + vector has been used for isolating allelic replacement mutations and for mutagenesis by directed plasmid integration in several different Gram-positive bacteria including *L. lactis* (Biswas et al., 1993), *Streptococcus pyogenes* (Perez-Casal et al., 1994) and *S. aureus* (Supersac et al., 1998). This is the first report of mutagenesis with this vector in *S. epidermidis*. Success was possible because of our ability to mobilize the plasmid from *S. aureus* to strain 9142 by pGO1-

**DISCUSSION**

A major problem in performing genetic manipulation in *S. epidermidis* is the difficulty in introducing plasmids into this organism by transformation. Here we used a conjugative plasmid, pGO1, to promote mobilization of the Ts vector pG + to strain 9142 was limited to three of the ten tested (data not shown).

The major conclusion to be drawn from this paper is that the Fbe (SdrG) proteins of *S. epidermidis* HB and K28 are surface proteins that promote adherence of *O. HARTFORD and OTHERS*

![Fig. 6](image-url) (a) Adherence of wild-type *S. epidermidis* HB (●) and the fbe mutant (■) to immobilized fibrinogen. (b) Adherence of *L. lactis* MG1363 (●) and MG1363 bearing pK580(fbe) cloned from strain HB (○) to immobilized fibrinogen.

2550
promoted conjugation. One advantage of using pG-Host9 to create insertion mutations is the stability of the integrated plasmid after prolonged growth at 37 °C in the absence of drug selection. This offers a considerable advantage over some other Ts plasmids whose mutant Rep proteins become active at that temperature. It is possible that pG-Host integrants would be stable during bacterial growth in experimental infections and mutations caused by plasmid integration could be used for analysing virulence.

ACKNOWLEDGEMENTS

We would like to thank Emmanuelle Maguin and Deitrich Mack for a generous gift of strains, Kirk McCrea for anti-SdrG antibodies and Magnus Höök for advice. This work was supported by The Wellcome Trust (grant number 052320), Inhibitex Inc. and BioResearch Ireland.

REFERENCES


Received 5 February 2001; revised 22 May 2001; accepted 23 May 2001.