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Asunto Solicitud de patente para **INMUNIZACIÓN DE GANADO LECHERO CON PROTEINA GapC CONTRA LA INFECCIÓN DEL ESTREPTOCOCOS**

Solicitante UNIVERSITY OF SASKATCHEWAN

Fecha de solicitud 12 de diciembre de 2002

1 Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al oficio numero **08081** notificado por fijación en lista del 28 de julio de 2006, y atentamente presento junto con este escrito los siguientes artículos

- **K. GASE ET AL** "*Cloning, sequencing and multifunctional overexpression of the Streptococcus equisimilis H46A gapC gene encoding a glyceraldehyde-3-phosphate dehydrogenase that also functions as a plasmin(ogen)-binding protein Purification and biochemical characterization of the protein*" EUROPEAN JOURNAL OF BIOCHEMISTRY, Vol 238, No 1, July 1, 1996, pages 42-51, XP001053701 Berlin, Germany

- **J. FINCH ET AL** "*Further studies on the efficacy of a live vaccine against mastitis caused by Streptococcus uberis*" VACCINE, Vol 15, No 10, July 1997, pages 1138-1143, XP004085975 Guildford, GB

3 Puesto que se han atendido a cabalidad las peticiones hechas por su Despacho sobre la presente solicitud de patente, atentamente solicito que se continúe su tramitación

Bogotá,
Señora Jefe,



EMILIO FERRERO

T P A No 31 929

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Cris M 2006-00012054

Cloning, sequencing and functional overexpression of the *Streptococcus equisimilis* H46A gapC gene encoding a glyceraldehyde-3-phosphate dehydrogenase that also functions as a plasmin(ogen)-binding protein

Purification and biochemical characterization of the protein

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We previously identified DNA sequences involved in the function of the complex promoter of the streptokinase gene from *Streptococcus equisimilis* H46A a human serogroup C strain known to express this gene at a high level. As a prerequisite to understanding possible mechanisms that control the balance between the plasminogen activating and plasmin(ogen) binding capacities of H46A we describe here its gapC gene encoding glyceraldehyde-3-phosphate dehydrogenase (GraP DH EC 1.2.1.12) a glycolytic enzyme apparently transported to the cell surface where it functions as a plasmin(ogen) binding protein. The gapC gene was cloned and sequenced and found to code for a 336-amino-acid polypeptide (≈ 35.9 kDa) exhibiting 94.9% sequence identity to the Pir protein from *Streptococcus pyogenes* shown by others to be capable of plasmin binding [Loitenberg, R., Bruder C. C., Boyle, M. D., Kun, S. J., Schroeder B. L. & Curtiss, R. III (1992) *J. Bacteriol.* 174, 5204–5210]. To study the properties of the GapC protein its gene was inducibly overexpressed in *Escherichia coli* from QIAexpress expression plasmids to yield the authentic GapC or (His)₆GapC carrying a hexahistidyl N-terminus to permit affinity purification. Both proteins were functionally active, exhibiting specific GraP DH activities of about 80 kat/mol (≈ 130 U/mg) after purification. Their binding parameters [association (k_a) and dissociation (k_d) rate constants, and equilibrium dissociation constants ($K_d = k_d/k_a$)] for the interaction with human Glu-plasminogen and plasmin were determined by real time, biospecific interaction analysis using the Pharmacia BIAcore instrument. For comparative purposes, the commercial GraP DH from *Bacillus stearothermophilus* (BstGraP DH), a nonpathogenic organism, was included in these experiments. The K_d values for binding of plasminogen to GapC, (His)₆GapC and BstGraP DH were 220 nM, 260 nM and 520 nM, respectively as compared to 25 nM, 17 nM and 98 nM, respectively, for the binding to plasmin. These data show that both the zymogen and active enzyme possess low-affinity binding sites for the gapC gene product and that the hexahistidyl terminus does not affect its function. Prior limited treatment with plasmin enhanced the subsequent plasminogen binding capacity of all three GraP DHs, presumably by the exposure of new C-terminal lysine residues for binding to the zymogen.

Keywords: *Streptococcus equisimilis*, glyceraldehyde-3-phosphate dehydrogenase gapC gene, plasmin (ogen)-binding protein.

As a major surprise of recent investigations into the nature of cell surface proteins of the pathogenic streptococci, a wall-associated plasmin binding protein has been discovered that structurally and functionally belongs to the glyceraldehyde 3-

phosphate dehydrogenase (GraP-DH) family of enzymes. Broder et al. (1991) were the first to purify from the surface of a group A streptococcal strain a ≈ 41 -kDa high-affinity plasmin binding protein with no affinity for Glu-plasminogen. The gene, designated *plr* for this plasmin receptor was subsequently cloned, expressed in *Escherichia coli* and shown to encode a 336-amino-acid polypeptide with 39–54% residue identity to known GraP-DHs (Loitenberg et al., 1992a). A similar *Streptococcus pyogenes* surface protein with significant N-terminal sequence identity to pro- and eukaryotic GraP-DHs was identified by Pancholi and Fischetti (1992) and shown to exhibit both GraP-DH enzymatic activity and multiple binding specificity for various mammalian proteins, including weak plasmin binding activity. Subsequently the same investigators reported that this GraP-DH, like several eukaryotic GraP-DHs (Kots et al. 1992; Zhang and Snyder 1992), has also ADP-ribosylating activity (Pancholi and Fischetti 1993).

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Abbreviations: BHI brain heart infusion; GraP-DH generic abbreviation for glyceraldehyde-3-phosphate dehydrogenases; BstGraP DH GraP DH from *Bacillus stearothermophilus*; IPTG isopropyl β -D-thiogalactopyranoside; LDS lysine binding site; NTA, Ni²⁺ nitrilotriacetic acid; D Val-Leu-Lys-CH-Cl, D-valyl-leucyl lysyl-chloromethane; Enzymes, Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) plasmin (EC 3.4.21.7).

Note: The novel nucleotide sequence data published here has been deposited with the EMBL/GenBank/DBJ sequence data banks and is available under accession number X97788.

It is intriguing that these streptococcal cell surface GraP DHs are distinct from other wall-associated proteins of gram-positive cocci (Fischetti et al., 1991) in that they lack an N-terminal signal peptide, tandemly repeated regions, and known wall-associating signals. Thus, the mechanisms underlying their secretion and anchoring to the cell surface are presently not understood. Neither is their functional significance. Primarily, GraP-DHs are intracellularly located glycolytic enzymes. However, several prokaryotic and eukaryotic organisms are known to harbor not only multiple GraP-DH genes but also isoenzymes with alternative locations and functions [see Lottenberg et al. (1992a) and Pancholi and Fischetti (1992) for a literature survey]. Thus the GraP-DH enzyme family appears to be involved in a variety of functional activities and as surface proteins of the pathogenic streptococci, may contribute to virulence by virtue of their binding activities to various host proteins. Among these, the plasmin(ogen) binding capacity appears to be of special interest in view of the fact that essentially all group A C and G hemolytic streptococci produce the potent plasminogen activator streptokinase (Huang et al. 1989), thereby generating the ligand to be captured by appropriate plasmin receptors on the cell surface. In fact evidence has been provided to show that when growing or heat-killed *S. pyogenes* cells are incubated in human plasma, their ability to acquire cell-associated plasmin activity depends on the simultaneous presence of active streptokinase (Lottenberg et al., 1992b). In addition, using the wild-type *S. equisimilis* strain H46A and an isogenic mutant carrying an inactivated streptokinase gene, we have shown that H46A binds both the zymogen and active enzyme, generates surface-associated plasmin activity when producing streptokinase, and expresses its plasmin(ogen) receptor(s) independently of a functional streptokinase gene (Malke et al., 1994).

Strain H46A of *S. equisimilis* is known to be a particularly potent streptokinase producer (Christensen, 1945) and recent studies of the expression control of its streptokinase gene, *ske*, have revealed a complex promoter structure consisting of two tandemly oriented core promoters with different activities and an upstream region which strongly contributes to full *ske* expression (Gase et al. 1995; Gräfe et al. 1996). As a further step towards elucidating regulatory relationships between elaboration of streptokinase and acquisition of cell-associated plasmin activity we have now cloned, sequenced and overexpressed the GraP DH gene (*gapC*) of strain H46A and report kinetic parameters of the purified GapC protein that relate to its GraP DH enzymatic and plasmin(ogen) binding activities.

MATERIALS AND METHODS

Materials. Biochemicals were from Bachem, Boehringer Mannheim, Pharmacia or Sigma. Restriction enzymes, T4 DNA ligase and Vent DNA polymerase were obtained from New England Biolabs. The DNA sequencing kit and [³²S]dATP[αS] (37 TBq/mmol) were purchased from United States Biochemical Co. and Amersham, respectively.

Ni²⁺-nitrilotriacetic acid (Ni-NTA) linked to agarose was purchased as part of the QIAexpress system from Qiagen (Düsseldorf). Polyvinylidene difluoride membranes were from Millipore. BstGraP-DH (50–150 U/mg protein corresponding to a molar activity of 30–90 kat/mol) plasmin and lysozyme were from Sigma. Human plasminogen was purified according to the method of Deutsch and Mertz (1970).

Bacterial strains and culture conditions. Strain *S. equisimilis* H46A, a human group C isolate (Christensen 1945) was the source of chromosomal DNA for the polymerase chain reaction (PCR) and also the target strain for genomic plasmid integ-

ration. H46A was grown without agitation at 37°C in brain-heart infusion (BHI) medium (Difco). If appropriate, BHI was supplemented with erythromycin (2.5 µg/ml) to select for plasmids or glycine (20 mM) to facilitate electrotransformation. Most plasmid constructions and overexpression of the *gapC* gene were done in *E. coli* TG1 [*supE* Δ(*hsdM-mcrB*)5 *thi* Δ(*lac proAB*)F'⁺(*trc*Δ36 *proA*⁺*B*⁺ *lac*⁺ ZΔ M15)] (Carter et al., 1985). The gene for (His)₆GapC, designated *gapC*_{His}, was constructed and overexpressed in *E. coli* M15 (Str⁺ *lacZ*⁺) (Zamenhof and Villarejo, 1972). *E. coli* strains were grown in 2xYT medium (Sambrook et al. 1989) supplemented with ampicillin (100 µg/ml), kanamycin (40 µg/ml) or erythromycin (200 µg/ml) to select for plasmids.

Recombinant DNA techniques. Recombinant DNA procedures were performed as recommended by the manufacturers of the enzymes or according to standard protocols (Sambrook et al., 1989). Southern hybridization of restricted H46A chromosomal DNA was performed as described previously (Frank et al. 1995). The PCR was performed with 1 µg template DNA, 3 µM of each primer, 400 µM of each dNTP, 4 mM Mg²⁺, and 2 U Vent polymerase over 30 cycles (1 min each at 94°, 45° and 74°C) in a total volume of 50 µl. DNA was sequenced on both strands by the dideoxynucleotide chain termination method with double-stranded DNA serving as template. DNA primers were synthesized on an Applied Biosystems 394A DNA synthesizer or purchased from Pharmacia Biotech. The following oligonucleotides, listed in 5'-3' direction, served as PCR primers: N1, ggcgcggatcttagggagggtct**ATGGTAGTTAAAGTTGGTATTAACG**; N2, ggcgcggatcttagggagggtct**ATTTAGCAATTTTTCG**; N3, cgcgcggatcttagggagggtct**ATTTAGCAATTTTTCG**. Capital letters indicate identity to the *gapC* sequence as shown in this paper. The translation initiation codon and complement of the termination codon are underlined, and restriction sites are in bold face. N1 and N2 correspond to the 5' and 3' coding regions respectively, of the plasmin receptor gene (*plr*) from group A streptococcal strain 64/14 (Lottenberg et al., 1992a). The N1 nucleotides indicated in italics correspond to the 5' end of the *S. equisimilis* streptokinase gene (Malke et al. 1985) and served to provide a ribosome binding site (*aggagg*) known to function well in *E. coli* (Gase et al., 1995). To facilitate cloning, a *Bgl*II site was introduced at the 5' end of N1 and *Eco*RI and *Sal*I sites were incorporated at the 5' end of N2. N3 corresponds to the 5' coding region of *gapC*, with the translation initiation codon being replaced by a triplet that is part of an introduced *Bam*HI site (see Fig. 1).

Isolation of chromosomal DNA from *S. equisimilis* H46A. Cells were lysed as described elsewhere (Gase et al., 1995), with the following modifications. BHI cultures (25 ml) grown to an A₆₀₀ of 1.0 were pelleted and resuspended in 1.5 ml ice-cold lysis buffer I (10% sucrose, 0.1 M NaCl, 0.1 M EDTA, pH 8.0 containing 4 mg/ml lysozyme) and incubated for 30 min at 0°C. After centrifugation, the pellets were resuspended in 400 µl lysis buffer II (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 100 mM sodium acetate), mixed with 50 µl 10% SDS, and incubated for 15 min at 70°C. After addition of 200 µl distilled water the cell lysate was incubated for 30 min at 37°C with 125 µg/ml RNase A and then for 30 min at 37°C with 250 µg/ml proteinase K. After phenol/chloroform extraction, the chromosomal DNA was precipitated, washed and resuspended in 500 µl distilled water.

Plasmid constructions. Taking advantage of the strong similarity of the 5' and 3' regions of *gapC* with those of *plr* from *S. pyogenes* (Malke et al. 1994), *gapC* was amplified from the chromosomal H46A DNA by PCR primed with N1 and N2. The ~1100-bp PCR product was inserted as *Bgl*II–*Eco*RI fragment into plasmid pSLG2 (Gase et al., 1995) to form pPRG10 (4.2 kb). In pPRG10 *gapC* was preceded by a unique *Bgl*II site

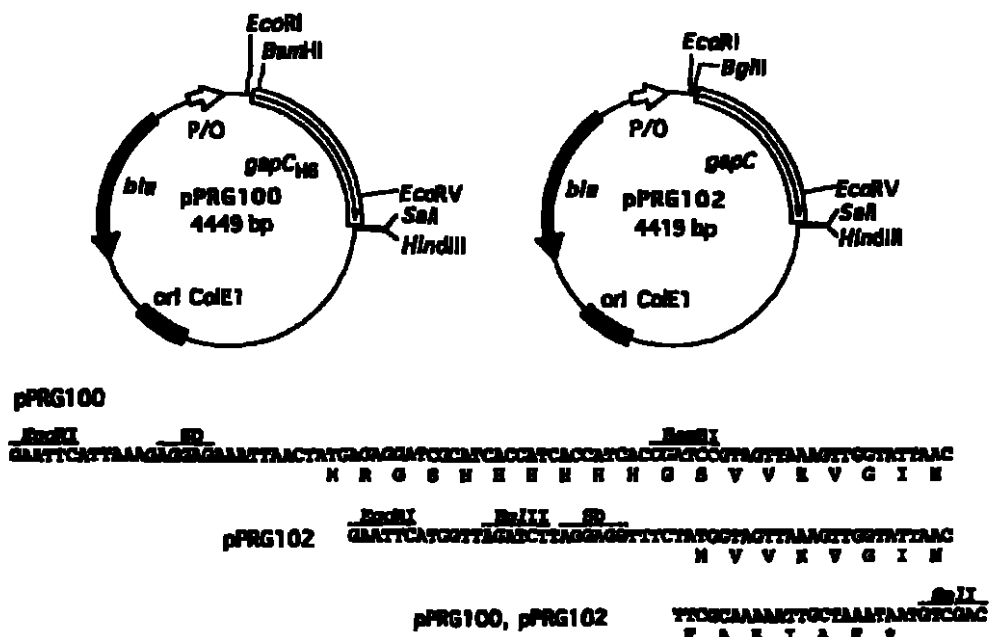


Fig. 1 Plasmids used for overexpression of *gapC* and *gapC_{His}*. pPRG100 contains *gapC_{His}* encoding six N-terminal histidine residues. *gapC_{His}* was overexpressed under control of the IPTG-inducible promoter/operator element (P/O) of pQE9 (Henco, 1991) to yield (His)₆GapC consisting of 347 amino acid residues. Authentic GapC (336 amino acid residues) was expressed from pPRG102 under the same control element. Detailed sequence information for the 5' and 3' regions of the *gapC* inserts in the two plasmids including relevant restriction sites and the ribosome binding site (SD) is given below the plasmid maps. For further details see Materials and Methods.

and the ribosome binding site (*rlc*, both encoded by N1. A unique *KpnI* site originating from pSLG2 was located 245 bp downstream of the *gapC* stop codon. Further bounded by the above restriction sites, pPRG10 contained the p15A origin of replication and the *ermAM* erythromycin resistance gene selectable in both *E. coli* and *S. equisimilis*. Using electroporation (Gase et al., 1995) followed by selection of erythromycin-resistant colonies, pPRG10 was integrated via *gapC* into the *S. equisimilis* chromosome, thus duplicating and genetically tagging the resident *gapC*. To clone *gapC* with its authentic 3' flanking region, H46A *gapC* pPRG10 chromosomal DNA was fragmented with *KpnI*. The fragments were circularized by ligation and transformed into *E. coli* TG1 resulting in plasmid pPRG23 (6.8 kb). Similarly using the *BglII* site for DNA excision, *gapC* with its authentic 5' flanking region was cloned on plasmid pPRG273 (6.0 kb).

To attempt insertional inactivation of the chromosomal *gapC* gene, four plasmids incapable of replication in H46A were constructed, each containing a specific *gapC* fragment. The 717 bp *BstYI* fragment of *gapC* was cloned in either orientation into the *BamHI* site of pVA8912 (Steiner and Malke, 1995) to form pPRG15 and pPRG16. The 658-bp *BstYI*–*HincII* fragment and the 204-bp *BstYI*–*AluI* fragment of *gapC* were also inserted into pVA8912 to yield pVAG2 and pVAG205 respectively. The recombinant plasmids were used for electroporation of H46A with selection for erythromycin-resistant transformants.

For overexpression of *gapC* in *E. coli*, the QIAexpress paired pREP4–pQE9 plasmid system was used (Henco, 1991). *gapC* was cloned into pQE9 as *BamHI*–*SalI* PCR fragment generated with the N2 and N3 primers, and pPRG10 as template yielding pPRG100 (4.4 kb, Fig. 1). After induction with isopropyl β -D-thiogalactopyranoside (IPTG; see below), *E. coli* M15(pREP4, pPRG100) overproduced GapC as N-terminal hexahistidyl derivative [(His)₆GapC] rendering possible its isolation by Ni-NTA affinity chromatography (Henco, 1991). To produce authentic GapC, the *EcoRI*–*EcoRV* fragment from pPRG100 containing the ribosome binding site and 5' region of *gapC_{His}* was exchanged for the corresponding *EcoRI*–*EcoRV*

fragment from pPRG10 to yield pPRG102 (4.4 kb, Fig. 1). From this plasmid, *gapC* was inducibly expressed in *E. coli* TG1(pREP4, pPRG102).

The sequence of *gapC* was determined in pPRG23, pPRG273 and, additionally, in pPRG101, a plasmid obtained by cloning the *gapC*-containing *BamHI*–*HincII* fragment from pPRG100 (Fig. 1) in pUC19. Two independent isolates of each pPRG23, pPRG273 and pPRG101 served for *gapC* sequencing and yielded identical results with sequencing primers including the standard pUC primers, N1, and, additionally, walking primers based on newly generated sequences.

Isolation of GapC and (His)₆GapC. At $A_{600} = 1$, 100-ml *E. coli* M15(pREP4, pPRG100) or TG1(pREP4, pPRG102) cultures were supplied with 1 mM IPTG and continued to be incubated at 37°C to $A_{600} = 4$. Cells were pelleted and resuspended in 5 ml 50 mM sodium phosphate pH 8.0 containing 0.3 M NaCl and for (His)₆GapC isolation, additionally 0.1% Tween 20. The cells were disrupted by sonication, the sonicates centrifuged for 30 min at 15000 g, and the pellets resuspended in 5 ml of the above buffers. The proportions of soluble and insoluble Gra⁺ DH activities were estimated by SDS/PAGE of aliquots taken from the supernatant and pellet fractions. (His)₆GapC was isolated from the supernatant fraction of M15(pREP4, pPRG100) by Ni-NTA affinity chromatography as described in the QIAexpress manual (Henco, 1991). Pooled (His)₆GapC-containing fractions and the supernatant fluid containing GapC were subjected to gel filtration on a calibrated HiLoad 16/60 Superdex 200 column in an FPLC system (Pharmacia) using procedures recommended by the manufacturer. Protein concentrations in pooled (His)₆GapC and GapC fractions analyzed by SDS/PAGE were estimated spectrophotometrically at 280 nm ($A_{280}^{1\%1\text{cm}} = 0.548$ and 0.573, respectively). Alternatively, protein concentrations were also determined by the biuret method according to the instructions of the manufacturer (Pierce Chemicals) and found to agree with those measured spectrophotometrically within errors of $\approx 20\%$. GapC (210 $\mu\text{g/ml}$) and (His)₆GapC (320 $\mu\text{g/ml}$) were stored in gel filtration buffer (20 mM sodium

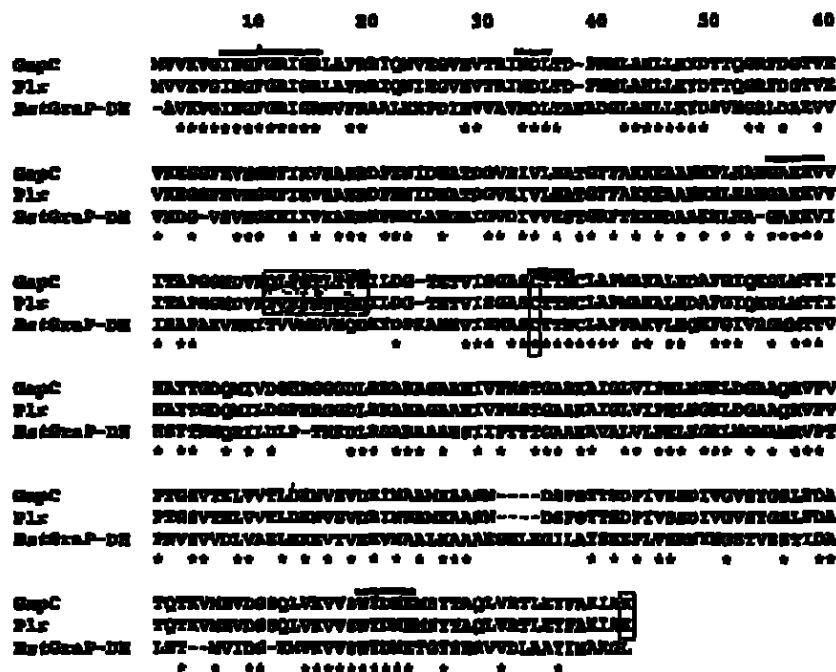


Fig. 3. Alignment of the amino acid sequences of the GrpP-DHs from *S. equisimilis* (GapC), *S. pyogenes* (Plr, Lottenberg et al., 1992a) and *B. steurothermophilus* (BatGrp-DH, Blescker et al., 1977). Identical and similar amino acids are marked by asterisks and dots respectively. The active-site cysteine residues and C-terminal lysine residues are boxed. The longest GapC and Plr segments that are most divergent are shaded. The five >3-residue segments that are completely conserved when 11 additional prokaryotic GrpP-DHs are included (see Cancilla et al., 1995) are overlined.

To ensure comparable conditions, the same sensor chips were exposed to different analytes in a fixed order, and the surfaces were regenerated after each change of analyte by injection of 15 µl 5 mM HCl at a flow rate of 5 µl/min. To begin with the virgin ligands were exposed to plasminogen. The next analyte was plasmin previously treated at 10 µM for 10 min at 37°C with a 200-fold molar excess of the plasmin inhibitor D-valyl-leuyl-lysyl-chloromethane (D-Val-Leu-Lys-CH₂Cl). This treatment is known to retain some residual proteolytic activity as indicated by an efficiency index of D-Val-Leu-Lys-CH₂Cl (k_2/K_1 , 100 M⁻¹ s⁻¹ where K_1 is the dissociation constant of the plasmin-inhibitor complex and k_2 is the alkylation rate constant) which is up to 20-fold lower than that of other chloromethane inhibitors (Collen et al., 1980; Lijnen et al., 1992). In the third cycle, the ligands previously treated with plasmin were again exposed to plasminogen to study possible alterations of the kinetics of zymogen binding following exposure to plasmin previously treated with D-Val-Leu-Lys-CH₂Cl. To examine the influence of 6-aminohexanoic acid on analyte binding, sensorgrams were recorded using plasmin and plasminogen at 200 nM and 5 µM, respectively in the following order of the analytes: plasmin, plasmin in the presence of 0.1 M 6-aminohexanoic acid, plasmin, plasminogen, plasminogen with 0.1 M 6-aminohexanoic acid, plasminogen. Previous exposure to plasmin plus 6-aminohexanoic acid did not alter the subsequent amount of plasmin binding in the absence of 6-aminohexanoic acid. Similarly, previous treatment with plasminogen plus 6-aminohexanoic acid did not alter the subsequent amount of plasminogen binding.

RESULTS

Nucleotide sequence of gapC and deduced protein sequence
An internal segment of gapC was cloned via PCR employing primers (N1 and N2) based on sequence data from the plr gene of *S. pyogenes* (Lottenberg et al., 1992a). The resultant eryth-

mycin-resistance plasmid pPRG10 was then inserted into the chromosome of H46A to tag the resident gapC and derive a set of recombinant plasmids containing authentic gapC segments for nucleotide sequencing and the construction of expression plasmids. The coding sequence of gapC (Fig. 2) comprises 1008 bp with a G+C content of 41.4%, which is slightly above the average of the *S. equisimilis* genome (37.3–40.0%, Ferretti and Curtiss III, 1987). Among the closest streptococcal homologs of gapC are the plr gene from *S. pyogenes* (Lottenberg et al., 1992a) and the gap gene from *Lactococcus lactis* (Cancilla et al., 1995) with which it shares 96.4% and 74.2% identity respectively. Immediately upstream of gapC, a well preserved ribosome-binding site was detected that showed a 9 bp spacing from the ATG start codon (Fig. 2). Further upstream, two putative -10 and -35 promoter regions were identified, with variable matches (3/6 to 6/6) to the *E. coli* σ⁷⁰ consensus promoter sequence. The available sequence upstream of the ATG start codon of plr (114 nucleotides, Lottenberg et al., 1992a) also shows a high degree of identity (95.6%) to the corresponding upstream sequence of gapC but ends within the -10 region of the potential upstream promoter identified for gapC. Downstream of the translational termination codon, TAA of gapC, there exists an inverted repeat (calculated ΔG of the potential stem-loop structure -38.5 kJ/mol) followed by an oligo(T) tract, features strongly suggestive of a factor-independent transcription terminator. The existence of potential promoter sequences and of a transcription termination signal is consistent with the detection by Northern hybridization of an ~1100-nucleotide gapC mRNA, indicating that the gene is monocistronically transcribed (Stümer K, Bruckmann A. and Malke H unpublished results). The available sequence 3' to the stop codon of plr (31 nucleotides exhibiting 61.3% identity to the corresponding gapC sequence) does not allow the identification of a potential transcription terminator.

The open reading frame of gapC encodes a 336-amino acid residue protein (calculated M_r = 35870) which shares 94.9%,

72.9% and 50.8% identity with its homologs from *S. pyogenes* (Lottunberg et al., 1992a) *L. lactis* (Cancilla et al. 1993) and *Bacillus steurothermophilus* (Biesecker et al., 1977), respectively (Fig. 3).

Failure to inactivate gapC by plasmid insertion Four plasmids (pVAG205, pVAG2, pPRG15 and pPRG16) carrying, respectively, 204-bp, 658 bp and 717-bp internal fragments of gapC (see Materials and Methods) were employed in transformation experiments of H46A aimed at insertionally inactivating the gene. Using procedures that previously led to the inactivation of other H46A genes (Mechold et al. 1993, 1996; Malke et al., 1994) or to plasmid insertion when the complete gapC gene was offered (see Materials and Methods), we failed to obtain any erythromycin-resistant transformants. This suggests that gapC is indispensable even when H46A is grown in complex medium. To substantiate this result, we subjected H46A DNA digested with several restriction enzymes to Southern hybridization using pPRG10 as gapC probe under stringent conditions as specified previously (Frank et al. 1995). For all enzymes or enzyme combinations used (*Hind*III, *Eco*RI, *Apa*I, *Hind*III + *Eco*RI, and *Hind*III + *Sa*I) the ³²P-labelled probe detected only single hybridizing fragments (approximate sizes 5.2, 2.2, 8.5, 2.1, and 2.2 kb, respectively). This is consistent with the sequence of gapC (Fig. 2) which lacks cleavage sites for each of the above enzymes. The sizes of the hybridizing fragments are also consistent with sequence data (not shown) indicating the presence of *Hind*III and *Eco*RI sites 540 and 610 bp, respectively downstream of the TAA stop codon of gapC. This observation also rules out the existence of two gapC genes arranged in tandem. Taken together, these findings indicate that there exists only a single copy of gapC in the *S. equisimilis* H46A chromosome.

Overexpression of gapC in E. coli and protein purification Of the two IPTG-inducible expression plasmids used, pPRG102 was designed to direct the synthesis of authentic GapC while pPRG100 specified a protein, designated (His)₆GapC, with a vector-encoded modified N-terminus (see Materials and Methods). Expected sequence, MRGSH (Fig. 1), including the first of the six histidine residues, was confirmed by microsequencing of the purified protein (calculated M = 37 138). In uninduced cultures of both producer strains TG1(pREP4, pPRG102) and M15(pREP4, pPRG100) gapC and gapC_{his} expression was tightly repressed whereas IPTG induction caused GapC and (His)₆GapC to become the most abundant proteins in total cell extracts (Fig. 4). As judged by SDS/PAGE and densitometry of the gels, about 40% of either protein were present in soluble form after sonication of the cells while the remainder was associated with the pellet fractions of the sonicates (Fig. 4, lanes 4 and 5). The total yield of the two proteins was about the same for both producer strains, amounting to approximately 240 µg/ml from cell suspensions of A₆₀₀ = 4 (corresponding to 4 mg *E. coli* cells). Ni-NTA affinity chromatography followed by gel filtration of (His)₆GapC resulted in a homogenous protein fraction (Fig. 4A, lane 7, Fig. 4B, lane 6) with a yield of 3.0 mg purified protein from 0.4 g *E. coli* cells. Purification of GapC resulted in a protein fraction contaminated with approximately 5% of a ~32-kDa protein of unknown origin (Fig. 4A, lane 6, Fig. 4B lane 7). In gel filtration GapC and (His)₆GapC migrated at positions corresponding to apparent molecular masses of more than 100 kDa, suggesting that the proteins occurred in oligomeric form.

Specific GraP-DH activities. The endogenous GraP-DH activity of supernatant fractions derived from sonicated cell suspen-



Fig. 4. Purification of GapC (A) and (His)₆GapC (B) produced in *E. coli* TG1(pREP4, pPRG102) and M15(pREP4, pPRG100), respectively, and monitored by SDS/PAGE analysis. Lane 1, crude extracts from cells without the expression vectors; lane 2, crude extracts from uninduced cells carrying the expression vectors; lane 3, crude extracts from cells induced by 1 mM IPTG; lanes 4 and 5, pellet and supernatant fraction, respectively, after sonication of IPTG-induced cells; lanes 6 and 7, soluble GapC fraction (3 µg protein) after gel filtration; lanes 8 and 9, soluble (His)₆GapC fraction (3 µg protein) after Ni-NTA affinity chromatography followed by gel filtration. Note the ~1.3-kDa molecular mass difference between GapC and (His)₆GapC, the position of which is indicated by asterisks on the right.

sions of A₆₀₀ = 10 (total protein, 1.65 mg/ml) was 70 nkat/ml for both *E. coli* TG1(pREP4, pQE9) and M15(pREP4, pQE9). In the presence of the IPTG-induced gapC and gapC_{his} expression plasmids (pPRG102 and pPRG100, respectively), the corresponding GraP-DH activities increased to 0.7 µkat/ml. Using these data together with the estimated yields of soluble protein from the expression systems (see above), the molar GraP-DH activity of GapC and (His)₆GapC in crude cell extracts was determined to amount to approximately 100 kat/mol. When determined immediately after purification, the molar GraP-DH activities of GapC and (His)₆GapC were about 80 kat/mol (i.e. ~130 U/mg). After two months of storage in 50% glycerol at -20°C, the activity of (His)₆GapC remained unaltered whereas that of GapC decreased to about 10%. One freeze-thaw cycle in the absence of glycerol reduced the activities of the two enzymes to 5% and 0.3%, respectively.

Association affinity constants of plasminogen and plasmin for binding to GapC and (His)₆GapC. Further characterization

Table 1 Affinity constants of plasminogen and plasmin for binding to GapC, (His)₆GapC and BstGraP-DH. Values given are means ± standard errors of at least five determinations. The means of the *K_i* values were derived from the *k₁* and *k₋₁* values of individual sensorgrams

Ligand	Analyte	10 ⁴ × <i>k₁</i>	10 ⁴ × <i>k₋₁</i>	<i>K_i</i>
		M ⁻¹ s ⁻¹	s ⁻¹	nM
GapC (His) ₆ GapC BstGraP-DH	plasminogen	14 ± 2.0	2.3 ± 0.12	220 ± 47
		11 ± 0.6	2.8 ± 0.11	260 ± 18
		5.6 ± 0.8	2.6 ± 0.18	520 ± 90
GapC (His) ₆ GapC BstGraP-DH	plasmin	280 ± 23	7.0 ± 0.55	25 ± 2.5
		200 ± 24	3.2 ± 0.21	17 ± 1.3
		120 ± 8.9	12 ± 2.3	98 ± 18
GapC (His) ₆ GapC BstGraP-DH	plasminogen following plasmin	24 ± 2.3	2.9 ± 0.3	122 ± 4.5
		21 ± 2.3	1.6 ± 0.17	80 ± 2.5
		12 ± 2.0	4.8 ± 0.41	434 ± 60

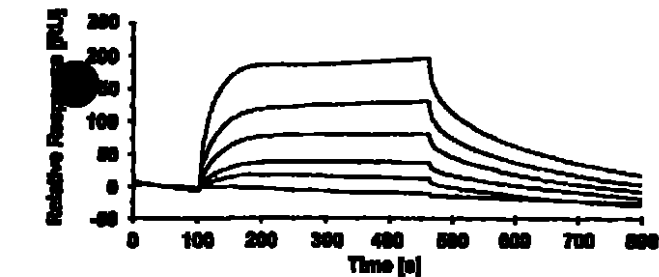


Fig. 5. Representative sensorgram depicting binding of plasmin (the analyte) at different concentrations (from top to bottom: 200, 100, 50, 25, 12.5, and 0 nM) to immobilized GapC (the ligand). The association phase began with plasmin injection at 100 s and the dissociation phase was started by buffer-alone flow at 470 s. Note the increasing resonance units (RU) with increasing analyte concentration. The very small response detected in the absence of the analyte (bottom curve) is due to the phosphate content of the buffer system.

of GapC and (His)₆GapC was aimed at determining the association rate constants (*k₁*), dissociation rate constants (*k₋₁*) and the equilibrium dissociation constants (*K_i* = *k₋₁*/*k₁*) for binding of plasminogen and plasmin to the two proteins. Included in these experiments was commercial GraP-DH from *B. stearothermophilus* (BstGraP-DH), an organism that underwent no evolutionary constraints towards binding the plasma proteins. The results are summarized in Table 1. A representative sensorgram is shown in Fig. 5 which demonstrates that the resonance response reflecting plasmin–GapC interaction occurred in an analyte concentration-dependent manner. No significant resonance signals were obtained from sensor chip surfaces without attached ligands (see Fig. 7D), indicating the absence of unspecific interactions between the sensor chip surfaces and the analytes. In all sensorgrams analyzed, the plots of ln(*R_t*/*R₀*) versus time (*t* – *t₀*), where *R₀* is the resonance signal at a fixed time (*t₀*) after the beginning of the dissociation phase and *R_t* the response at time *t*, were linear over a wide time interval. This indicates that dissociation followed homogeneous 1:1 dissociation kinetics. All three GraP-DHs included in these experiments were capable of binding both plasminogen and plasmin but did so with distinct rate constants. In general, the association rate constants of plasminogen for binding to GapC, (His)₆GapC and BstGraP-DH were lower than those for plasmin, differing for the two analytes by a factor around 20. GapC and (His)₆GapC showed very similar kinetic parameters in interactions with the plasma proteins, and these tended to bind with slightly higher affinities to the *Streptococcus* than the *Bacillus* GraP-DHs. Also the *K_i* values of the complexes between BstGraP-DH and the plasma proteins were significantly higher than the corresponding equilibrium

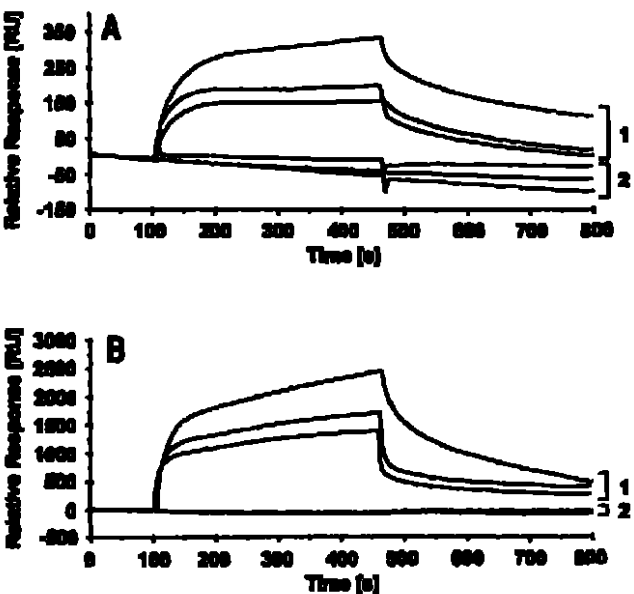


Fig. 6. Sensorgrams depicting blocking of the binding of plasmin at 200 nM (A) to (His)₆GapC, GapC and BstGraP-DH, and of plasminogen at 5 μM (B) BstGraP-DH, (His)₆GapC and GapC (from top to bottom) by 100 mM 6-aminohexanoic acid. The response curves obtained in the absence and presence of 6-aminohexanoic acid are marked by 1 and 2, respectively.

constants for GapC and (His)₆GapC, in the case of plasmin as a result of both a lower *k₁* and higher *k₋₁* value for BstGraP-DH. To further substantiate the specificity of the observed interactions and to assess the role of the lysine binding sites of plasminogen and plasmin in their interaction with the different GraP-DHs, we recorded sensorgrams in the presence of 100 mM 6-aminohexanoic acid, a lysine analogue that has higher affinity than lysine for these binding sites (Winn et al. 1980). As shown in Fig. 6, binding of both plasmin and plasminogen to all GraP-DHs was completely abolished by 6-aminohexanoic acid, indicating the involvement of lysine residues in the interactions.

Enhancement of plasminogen binding capacity of the GraP-DHs by prior treatment with plasmin. For all three GraP-DHs, sensor chips that had been exposed to increasing concentrations of plasmin (12.5–200 nM; see Materials and Methods) subsequently exhibited markedly increased total plasminogen binding compared to that shown by the virgin surfaces (Fig. 7). This enhancement of plasminogen binding as a result of previous exposure to plasmin was highest for BstGraP-DH (about 10-fold) followed by (His)₆GapC and GapC (about 6-fold and

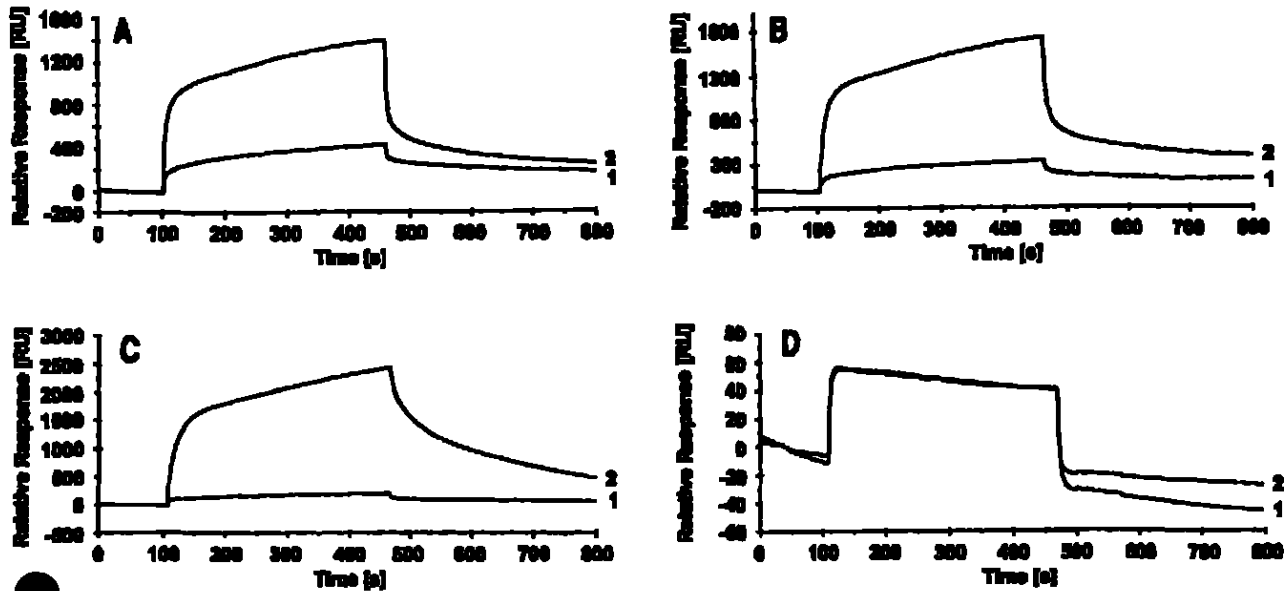


Fig. 7 Sensorgrams depicting the binding of plasminogen at 5 μ M to GapC (A), (His)₆GapC (B) and AsrGraP-DH (C) without (1) or with limited prior plasmin treatment (2) of the ligands. An empty sensor chip with no ligand attached was also included in this experiment (D)

4 fold, respectively) In contrast to stimulating the total plasminogen binding capacity prior treatment with plasmin did not substantially alter the kinetic parameters of subsequent plasminogen binding (Table 1)

DISCUSSION

Given the key role of GraP DH in glycolysis and its importance for molecular evolutionary studies (Doolittle et al 1990), the primary structures of the enzymes from many organisms have been reported (Rothergill-Gillmore and Michels, 1993) In this paper, we provide, on the basis of the cloned and sequenced *gapC* gene from *S. equisimilis*, the second amino acid sequence of a GraP-DH from a pathogenic streptococcal species Although *S. equisimilis* (serogroup C) and *S. pyogenes* (serogroup A) are well differentiated in overall chromosomal character (Bert et al, 1995) sequence comparison of their GraP DHs now shows that the enzymes share a high degree of residue identity (94.9%) Furthermore like *S. pyogenes* (Winram et al 1995), *S. equisimilis* also appears to carry a single copy of its GraP DH gene which seems to be indispensable. Apart from providing additional sequence information for comparative molecular analysis of related species, the availability of the *gapC* gene will allow its expression to be studied under different environmental conditions in relation to that of *skc* (Gase et al 1995 Gräfe et al 1996), which, together with *gapC*, determines the acquisition of cell surface associated plasmin activity in the H46A strain of *S. equisimilis* when grown in media containing only the zymogen (Malke et al., 1994).

The two *gapC* expression plasmids constructed (Fig. 1) proved to allow the inducible synthesis of the functional gene product at high levels in *E. coli* The specific activities of the enzymes expressed from pPRG102 (authentic GapC) and from pPRG100 [(His)₆GapC] were very similar and compared well with corresponding literature data (Ferdinand 1964, Branlant et al 1983) or the specific activity of commercial GraP DHs (see Materials and Methods) Furthermore, GapC and (His)₆GapC also showed similar affinities for plasminogen and plasmin (Table 1) These observations are important for several reasons They indicate that the modified N-terminus of the enzyme encoded by pPRG100 does not alter the functionality of the pro-

tein Thus, its (His)₆ tag allows it to be readily purified by one-step affinity chromatography, a circumstance that may greatly facilitate large scale functional analysis of mutant proteins in the future. In addition, the modified N-terminus appears to greatly increase the stability of the protein during storage, presumably by chelate formation with heavy-metal ions, trace amounts of which are known to inactivate the enzyme (Ferdinand 1964) The pPRG100 expression system, in contrast to the pPRG102 plasmid system, also enabled us to prepare a protein free of contaminating GraP-DH activity resulting from the functional *gapA* gene of the *E. coli* M15 strain

The present study is the first to use the surface plasmon response biosensor to obtain analytical binding data for the interaction between host and streptococcal cell surface proteins To derive such data, previous investigators usually relied on the Scatchard analysis of solid-phase binding assays, allowing whole cells or the coated wells of microtiter plates to interact with ¹²⁵I labeled tracer proteins (Broeseker et al., 1988, Carlsson-Wistedt et al 1995, Ullberg et al 1992). The present GapC/(His)₆GapC-plasmin(ogen) binding data are in contrast to those determined for the interaction of plasmin(ogen) with the closely related GraP DH (Pir protein) from *S. pyogenes* regarding both the specificity and the affinity of the interactions Pir showed no significant plasminogen-binding capacity and its affinity to plasmin ($K_d = 0.05$ nM) was found to be three orders of magnitude higher than that of GapC/(His)₆GapC (Lottenberg et al 1987, Broeseker et al 1988) Our GapC-plasminogen binding data are similar to those determined for an unidentified low-affinity plasminogen binding protein from group O streptococci (Ullberg et al 1992), and are close to those estimated for the low affinity binding sites ($K_d = 500$ nM) of certain M-like proteins from *S. pyogenes* (Carlsson Wistedt et al, 1995) They also fall well in the range of the dissociation constants for the plasminogen receptors of various mammalian cells (0.1–2 μ M, Miles and Plow, 1988) In view of the fact that GapC and Pir contain the same number of lysine residues at identical positions in the polypeptide chain (Fig. 3), the present conflict regarding their affinities to plasmin(ogen) is hard to explain Given the 2- μ M concentration of plasminogen in plasma, a K_d value around 0.2–0.3 μ M for the association of this protein with GapC (Table 1) might suggest that, within the vasculature, the great majority of the plasminogen-binding sites of *S. equisimilis*

H46A would be occupied. As shown previously by exploiting a mutant H46A strain carrying an insertionally inactivated streptokinase gene to eliminate endogenous plasminogen activating capacity, this streptococcal strain indeed binds plasminogen as demonstrated by the generation of cell surface-associated plasmin activity following exposure to exogenous plasminogen activators (Malke et al., 1994).

As might be expected, the binding of plasmin(ogen) to GapC(His)₆GapC can be blocked by 6-aminohexanoic acid, implying that lysine residues of the binding proteins are involved in the interaction with lysine-binding sites within the kringle domains of plasmin(ogen) (Winn et al., 1980). These sites preferentially recognize exposed C-terminal lysines so that probably many proteins fulfilling this premise can bind plasmin(ogen) (Miles et al., 1991; Plow et al., 1995). Consistent with this requirement, both Pir and GapC carry C-terminal lysine residues (Fig. 3), and the plasmin binding activity of Pir is greatly reduced when its terminal lysine is replaced by leucine (Winram et al., 1995). *AsrGraP-DH* lacks a C-terminal lysine residue (Fig. 3) but nevertheless, is capable of plasmin(ogen) binding albeit with lower affinities than GapC(His)₆GapC (Table 1). Most likely, exposed lysine residues other than C-terminal ones are also involved in the interaction. Evidence in support of this suggestion comes from a mutant Pir protein (Winram et al., 1995) with reduced plasmin binding activity in the presence of the C-terminal lysine but in the absence of the 33-amino-acid N-terminal segment containing a single lysine residue, which is also present in *BsrGraP-DH* and GapC (Fig. 3).

A novel aspect characterizing the interaction of plasmin(ogen) with the GraP DII's included in this study relates to our finding that limited prior treatment with plasmin greatly increases their subsequent plasminogen binding capacity (Fig. 7). Presumably the underlying mechanism involves the exposure of new plasminogen binding sites by the residual proteolytic activity of the D-Val-Leu-Lys-CH₂Cl-inhibited enzyme that, being a trypsin-like protease, preferentially catalyzes the hydrolysis of α -amino substituted lysine (and arginine) esters (Robbins and Summaria, 1970). Similar observations have been made with eukaryotic cells or their immobilized plasminogen receptor proteins (Amacho et al., 1989; Harpel et al., 1989; Gonzalez-Gronow et al., 1991; Plow et al., 1995). The pathogenic streptococci then have evolved a remarkable system to generate and amplify cell-surface protease activity. They manage to transport a common glycolytic enzyme to the cell surface where it functions as low-affinity plasminogen binding protein. The highly restricted function of streptokinase to form a complex with plasminogen capable of converting the zymogen to plasmin can now be exerted directly on the cell surface. Once activated, the cell-bound plasmin, being protected from inactivation by plasma inhibitors of free plasmin (Lottenberg et al., 1987; Plow et al., 1995), may serve to generate additional plasminogen binding sites on the GraP DH protein. While the generation of broad proteolytic activity on the cell surface may increase the invasiveness of the pathogen, it may also facilitate the destructive proteolysis of cell surface proteins like M proteins, that serve as virulence factors by inhibiting phagocytoses (Fischetti, 1989; Kehoe, 1994). Thus excessive proteolytic activity of the cell surface may be disadvantageous to the pathogen so that mechanisms controlling the balance between generating and limiting proteolysis may be operative. The analysis of the factors involved in the modulation of cell surface protease activity presents a challenge to what is presently understood of the plasminogen-activating and binding capacity of the pathogenic streptococci.

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Further studies on the efficacy of a live vaccine against mastitis caused by *Streptococcus uberis*

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Three groups of dairy cows were immunized by subcutaneous (s.c.) administration of a preparation of live *Streptococcus uberis* (strain 0140J) and an intramammary infusion of a soluble surface extract derived from same the bacteria. Animals in Groups 1 and 2 received two s.c. vaccinations plus an intramammary inoculation. Animals in Group 3 received two s.c. vaccinations but did not receive the intramammary infusion. In addition to the vaccinated animals, each group also contained two non-vaccinated (control) animals. All animals were challenged experimentally by intramammary infusion (in two quarters per animal) of ca 100 c.f.u. of *S. uberis* (strain 0140J or C221) and monitored for clinical signs of disease, bacterial numbers in milk, somatic cell count in milk, and daily milk yield for the following 10 days. Animals in Group 1 were challenged with strain 0140J. Only one out of six challenged quarters of three vaccinated cows developed clinical disease compared to all (four out of four) quarters of non-vaccinated cows. Animals in Group 2 were challenged with strain C221. All challenged quarters of three vaccinated (six out of six) and two non-vaccinated (four out of four) cows developed clinical mastitis. Animals in Group 3 were challenged with strain 0140J. Five out of eight quarters on four vaccinated cows developed clinical mastitis but the onset was delayed in comparison with that in both non-vaccinated cows in which four out of four challenged quarters developed clinical mastitis. These results indicated that vaccination with live *S. uberis* protects against challenge with the homologous strain but was less effective against a heterologous strain. Reduced protection was also seen when the intramammary booster was omitted.
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Keywords: bovine; mastitis; *Streptococcus uberis*

Streptococcus uberis is a common cause of bovine mastitis and is responsible for ca 20% of all clinical cases in the UK¹. Although control recommendations have effectively reduced the incidence of mastitis due to contagious pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*, they have had little impact on the incidence of mastitis caused by environmental pathogens such as *S. uberis*^{2,3}. Strategies based upon vaccination to prevent infection have therefore become highly desirable. Initial studies indicated that cows which had experienced a previous intramammary infection with *S. uberis* demonstrated a reduction in clinical mastitis following subsequent experimental challenge⁴. The mechanism by which this effect was attained was not known, although results obtained in other laboratories had indicated that enhanced levels of opsonic antibody and the rapid recruitment of neutrophils into the mammary gland are

required for protection of the mammary gland against infection by *S. aureus* and *Escherichia coli*⁵⁻⁷. Subsequent studies in which cattle were immunized with a subcutaneous (s.c.) preparation of live *S. uberis* (strain 0140J) plus an intramammary booster with bacterial surface extract derived from *S. uberis*, showed that protection could be achieved against experimental challenge with the same strain⁸. Protection against infection was achieved in the absence of opsonic antibody and without a marked influx of neutrophils into the mammary gland following challenge. The experiments reported in this communication were intended to determine the potential contribution of the intramammary booster to protection and to determine the extent of the protection against challenge with a non-vaccine strain of *S. uberis* (strain C221).

MATERIALS AND METHODS

Animals

Sixteen Friesian dairy cows from the Institute's dairy herd and with no history of mastitis caused by *S. uberis*

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were divided into three groups on the basis of their expected calving date. Groups 1 and 2 comprised three animals which received appropriate vaccinations and two non-vaccinated, controls, whilst Group 3 comprised four vaccinates and two non-vaccinated controls

Bacteria

S. uberis strains 0140J and C221 (originally isolated from cases of bovine mastitis) were used as indicated. Both organisms were stored in Todd Hewitt broth (Oxoid) at -20°C in the presence of 25% (v/v) glycerol. For inoculation of chemically-defined medium or infection of cattle, bacteria were grown in Todd Hewitt broth at 37°C for 18 h.

Vaccine preparations

Live *S. uberis* strain 0140J and bacterial surface extract were prepared as described by Hill *et al.*⁸ Briefly, bacteria were inoculated into chemically-defined medium⁹, grown overnight at 37°C , harvested during late exponential growth and washed twice in phosphate buffered saline (PBS). Live bacteria were then resuspended in 10% of the original culture volume to give a final concentration of 10^{10} c.f.u. ml⁻¹ and stored at -70°C until required. Bacterial surface extract was prepared from the bacterial suspension using mutanolysin and type X hyaluronidase as described by Hill *et al.*⁸ Resulting protoplasts were removed by centrifugation (7000g, 20 min) and the supernatant (extract) collected, sterilized by filtration and stored at -70°C until required.

Vaccination regime

Animals were vaccinated according to the published protocol⁸. Vaccinates in Groups 1 and 2 received two s.c. vaccinations [each comprising 10^{10} c.f.u. *S. uberis* strain 0140J plus 1 ml Freund's incomplete adjuvant and 2 ml 0.1% Tween 20, one 14 days before drying off and the second within 48 h of calving. These animals were also given an intramammary inoculation comprising 5 ml of the cell wall extract prepared from the same bacterial cells, into all four quarters 7 days into the dry period. This intramammary inoculation did not contain adjuvant material. Animals in Group 3 received the two s.c. vaccinations of live *S. uberis* (as above) but did not receive the intramammary infusion of cell wall extract during the dry period.

Experimental challenge

All animals were challenged with the appropriate strain of *S. uberis*, 2-6 weeks into lactation. Each animal received a 1 ml inoculum containing ca 100 c.f.u. in PBSa into each of two mammary quarters, whilst a third quarter was inoculated with 1 ml PBSa only. Sterile quarter milk samples were collected at each milking until the end of the 10 day experiment. The numbers of bacteria and somatic cells present in the milk were determined and the milk yields recorded. Any quarters displaying clinical signs (clotted/discooured foremilk, swollen and tender quarter) and known to be bacteriologically positive were given a course of antibiotic

therapy (Leo Yellow). Any infected quarters which had not already been treated were given a course of antibiotics at the end of the experiment

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Determination of *S. uberis* specific antibody in serum and milk

Levels of specific antibody in serum and milk were determined by ELISA⁸. Briefly, microtitre plates were coated with the bacterial suspension (above) diluted in 0.05 M carbonate buffer, blocked with 1% (w/v) skimmed milk, and serum and milk samples added to appropriate wells. Mouse anti-bovine isotype specific monoclonal antibodies were then added and visualized by the addition of biotinylated goat anti-mouse IgG followed by streptavidin-HRP. Plates were developed using 3,3',5,5' tetramethylbenzidine (TMB, Kirkegaard and Perry Labs Inc.), the reaction was stopped by the addition of 1 M sulphuric acid and the changes in optical density measured at 492 nm using an ELISA plate reader. Samples of serum and milk were obtained prior to vaccination and post-vaccination (immediately prior to challenge).

RESULTS

Group 1. Subcutaneous and intramammary vaccination with *S. uberis* strain 0140J followed by challenge with the same strain

Vaccinates in Group 1 had been immunized with live *S. uberis* strain 0140J plus the intramammary booster and were challenged with the homologous strain. Bacteria were recovered in large numbers from the milk of all four quarters on two non-vaccinated animals (*Figure 1a*). Clinical signs were evident in three of these quarters within 48 h of challenge and were treated with antibiotic at the next milking. The fourth quarter did not display any clinical signs until 7 days post-challenge and was treated with antibiotic at milking 15. In contrast, *S. uberis* was never recovered from three of the six challenged quarters on vaccinated animals and the mean numbers of bacteria recovered were very low (*Figure 1a*). Two quarters on a single animal shed low numbers of bacteria for a short time before clearing the infection in the absence of any clinical signs. Bacteria were also recovered from a single quarter of another vaccinated animal although the number recovered was reduced during the first seven milkings post-challenge when compared to those from non-vaccinated animals. Clinical signs were not evident in this quarter until milking 11 and at this time the quarter was treated with antibiotics.

Mean somatic cell counts increased markedly in control animals within 48 h of challenge (*Figure 1b*), whilst only a slight rise in mean cell count was seen in the milk of vaccinated cows. Somatic cell counts were elevated in all quarters from which bacteria were recovered, although in the case of vaccinated animals, these rises were much less than that seen in controls, the increase was transient and followed the recovery of bacteria from the milk (*Figure 1b*). The dramatic fall in the milk yield of control animals (*Figure 1c*) coincided with the onset of clinical signs whilst the mean milk yield in vaccinated animals did not fall below 85% of that prior to challenge.

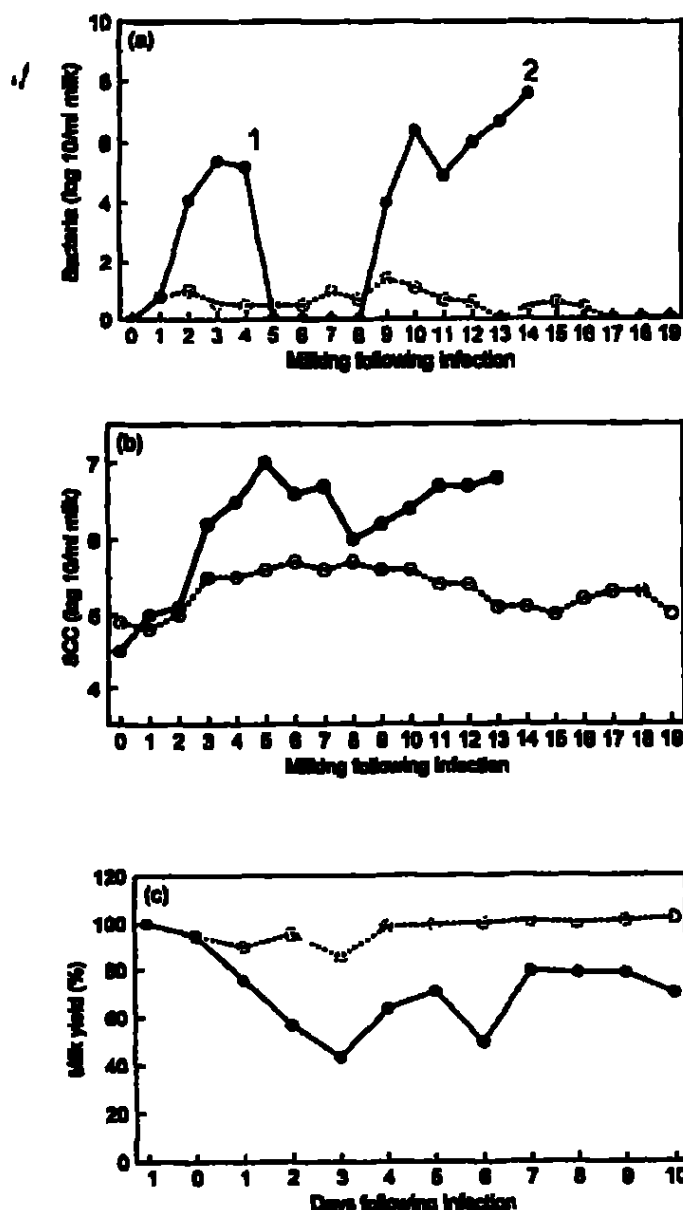


Figure 1 Mean bacterial recovery (a), somatic cell count SCC (b) and alterations in milk yield (c) of control (—) and vaccinated (---) cows in Group 1. Vaccinates were immunized with *S. uberis* strain 0140J and all cows were challenged with strain 0140J during lactation. ¹Four quarters on two control cows, ²six quarters on three vaccinated cows. ³Three out of four quarters treated, ⁴remaining quarter treated

Group 2. Subcutaneous and intramammary vaccination with *S. uberis* strain 0140J and challenge with strain C221

Vaccinates in Group 2 had been immunized with live *S. uberis* strain 0104J plus the intramammary booster but were challenged with ca 100 c.f.u. *S. uberis* strain C221. Bacteria were recovered in large numbers from the milk of all four quarters on two non-vaccinated cows (Figure 2a), one quarter required antibiotic therapy at milking 4, although clinical signs in the three remaining quarters only became evident around the 12th milking post-challenge. By comparison, the mean recovery of *S. uberis* was lower in the challenged quarters on vaccinated cows for the first three to six milkings post-infection (Figure 2a). By milking 5, only one out of six challenged quarters had developed clinical signs of

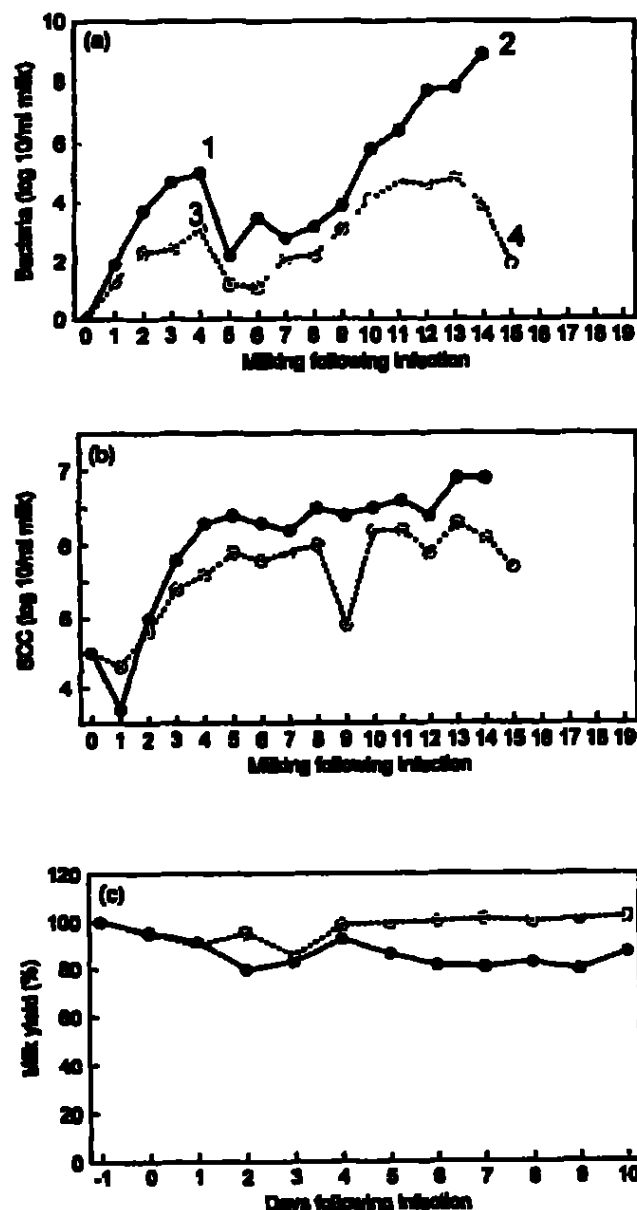


Figure 2 Mean bacterial recovery (a), somatic cell count SCC (b) and alterations in milk yield (c) of control (—) and vaccinated (---) cows in Group 2. Vaccinates were immunized with *S. uberis* strain 0140J and all cows were challenged with strain C221 during lactation. ¹Four quarters on two control cows, ²six quarters on three vaccinated cows. ³One out of four quarters treated, ⁴three remaining quarters treated, ⁵one out of six quarters treated, ⁶five remaining quarters treated

mastitis and this quarter required treatment at this time. Subsequently, the mean bacterial numbers gradually increased until they became sufficient to induce clinical signs and the five remaining quarters were treated at milking 15.

Somatic cell counts were elevated in all quarters from which bacteria were recovered in both vaccinated and non-vaccinated animals and there was little difference in the mean cell counts of the two groups (Figure 2b). The mean milk yield fell by 20% in non-vaccinated animals challenged with strain C221 (Figure 2c), compared with a fall of around 60% in similar animals challenged with strain 0140J. An additional fall in milk yield was observed in one cow 6–7 days post-challenge; this coincided with a loss of condition and appeared not to be

directly related to the experimental infection. The mean milk yield of vaccinated cows fell to 80% of that recorded prior to challenge (Figure 2c) and this fall was mainly a result of the clinical infection in the single quarter of one vaccinated cow

Group 3. Subcutaneous vaccination and challenge with *S. uberis* strain 0140J

The vaccinates in Group 3 had been immunized with live *S. uberis* strain 0104J but did not receive the intramammary infusion of cell surface extract 7 days into the dry period. These cows were subsequently challenged with ca 100 c.f.u. *S. uberis* strain 0140J in order to determine the importance of the intramammary vaccination in inducing the level of protection attained in Group 1.

Bacteria were recovered in large numbers from the milk of all four quarters of non-vaccinated cows (Figure 3a) and clinical signs became evident within 24 h post-challenge. All quarters from these animals had received antibiotic therapy by the end of milking 6. By comparison, there was a marked reduction in the mean numbers of bacteria recovered from the milk of vaccinated cows (Figure 3a). Three of the eight challenged quarters on vaccinated cows shed only low numbers of bacteria and two of these quarters cleared the infection without displaying any clinical signs or need for antibiotic therapy. The number of bacteria recovered from the five remaining quarters did, however, increase following challenge such that all quarters developed clinical signs between milking 4 and milking 10 and these quarters required antibiotic therapy.

As in Groups 1 and 2, somatic cell counts were elevated in all quarters from which bacteria were recovered and there was little difference in the magnitude of the mean counts of the control and vaccinated cows (Figure 3b). The milk yield of control animals was depressed by 40% 2–3 days post-challenge but recovered following antibiotic therapy (Figure 3c), whilst that of vaccinated cows fell by only 10–20% following challenge.

Levels of *S. uberis* specific antibody in serum and milk

Mean levels of *S. uberis* specific IgG1 and IgG2 were increased in the serum of vaccinated animals to between 2- and 20-fold of that seen prior to vaccination. Those in non-vaccinated animals remained low. None increased more than threefold during the period prior to challenge (Table 1). In contrast, there was relatively little change in the mean levels of specific IgG1 or IgG2 in the milk of vaccinated cows (Table 2) and with the exception of the levels of IgG2 in Group 2 and the levels IgG1 in Group 3 there was no discernable elevation in the local antibody responses of vaccinated animals. The levels of IgG1 and IgG2 in milk from non-vaccinated animal remained similar throughout this period (Table 2).

DISCUSSION

Results obtained from the animals in Group 1 confirmed the effect of the published vaccination protocol comprising parenteral vaccination of dairy cows with live *S. uberis* plus an intramammary infusion of bacterial surface extract⁸, and showed that this was capable of

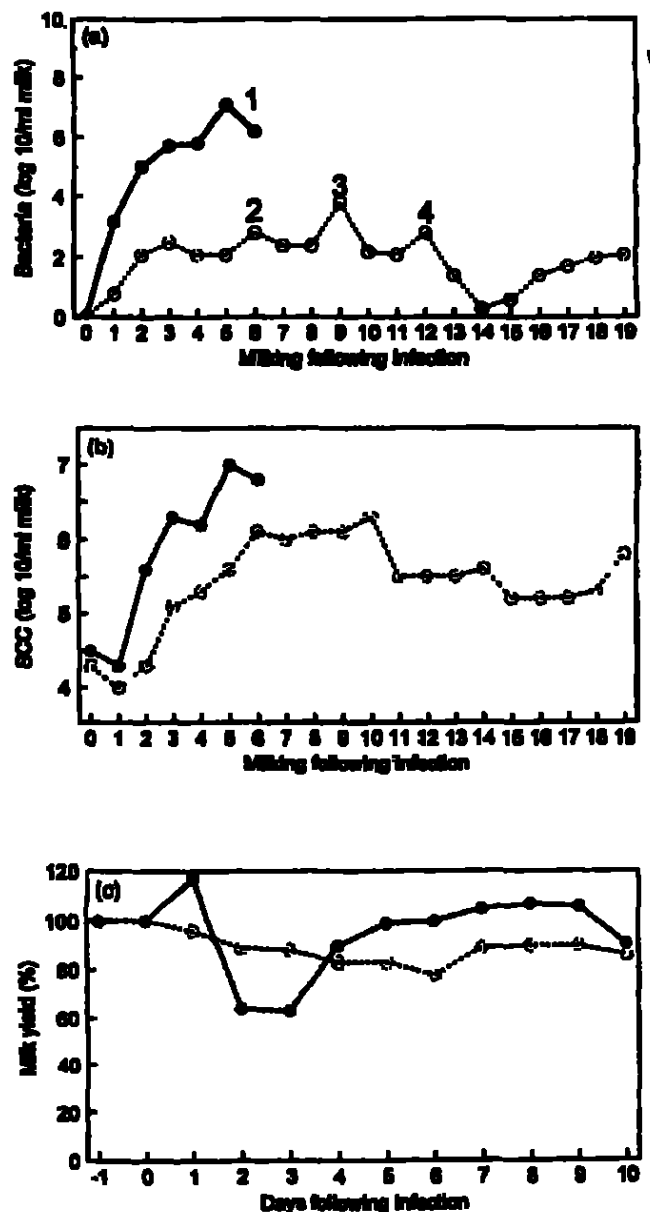


Figure 3. Mean bacterial recovery (a), somatic cell count (b) and alterations in milk yield (c) of control (—) and vaccinated (---) cows in Group 3. Vaccinates were immunized with *S. uberis* strain 0140J but did not receive the intramammary booster, and all cows were challenged with strain 0140J during lactation. *n*=Four quarters on two control cows, *n*=eight quarters on four vaccinated cows. ¹All four quarters treated, ²one out of eight quarters treated, ³two out of seven remaining quarters treated, ⁴two out of five remaining quarters treated.

protecting the mammary gland from subsequent experimental challenge with the same strain. These results consolidate and, in fact, supersede those obtained in the previous study since the difference between controls and vaccinates for all the parameters measured (bacterial recovery, somatic cell counts, alterations in milk yield and clinical signs) was much greater. This suggests that s.c. immunization with a live, or possibly an attenuated live, vaccine may be a viable proposition for the prevention of mastitis caused by this organism.

The clinical and bacteriological data obtained from Group 2 following the challenge of vaccinated animals with heterologous strain C221 suggests that the initial colonization of the mammary gland by *S. uberis* strain C221 was delayed in vaccinated animals when compared

Table 1 Mean levels of *S. uberis* specific IgG1 and IgG2 in serum

<i>S. uberis</i> specific antibody in serum			
	Pre-vaccination	Pre-challenge	Fold increase
IgG1			
Group 1			
Controls	4828	7436	1.51
Vaccinates	10202	31414	3.08
Group 2			
Controls	6884	13839	2.35
Vaccinates	6180	122313	19.79
Group 3			
Controls	11843	13504	1.20
Vaccinates	8575	172185	20.08
IgG2			
Group 1			
Controls	11388	13823	1.22
Vaccinates	14115	59783	4.23
Group 2			
Controls	41717	32165	0.77
Vaccinates	30480	81883	2.68
Group 3			
Controls	45210	63700	1.41
Vaccinates	27185	228813	8.34

Table 2 Mean levels of *S. uberis* specific IgG1 and IgG2 in milk (values represent the mean of samples collected from all four quarters of each animal)

<i>S. uberis</i> specific antibody in milk			
	Pre-vaccination	Pre-challenge	Fold increase
IgG1			
Group 1			
Controls	134	87	0.72
Vaccinates	1688	684	0.41
Group 2			
Controls	1185	412	0.35
Vaccinates	257	351	1.38
Group 3			
Controls	287	183	0.64
Vaccinates	513	2882	5.18
IgG2			
Group 1			
Controls	24	45	1.87
Vaccinates	141	302	2.14
Group 2			
Controls	113	171	1.51
Vaccinates	97	8483	86.80
Group 3			
Controls	183	103	0.58
Vaccinates	111	188	1.70

with controls and that the mean numbers of bacteria recovered remained low ($<10^3$ c.f.u ml⁻¹ milk) for up to seven milkings post-challenge before they finally attained sufficient numbers to cause clinical disease. It was also noted that clinical signs in both control animals and one of the three vaccinates were more extreme than those in the two remaining vaccinates and this appeared to be related to the very high numbers of bacteria recovered from these quarters. These results indicate that vaccination with *S. uberis* strain 0140J may retard bacterial colonization following challenge with a heterologous strain, but that this is insufficient to prevent

infection and thereby reduce the incidence of clinical disease. An effective live vaccine may therefore require a cocktail of several different strains of *S. uberis* in order to achieve protection against *S. uberis* mastitis in the field. It should be borne in mind, however, that this is based upon data from challenge with only a single heterologous strain and that protection against other less virulent strains may have been achieved.

The results obtained from Group 3, in which the intramammary infusion of bacterial surface extract during the dry period was omitted, revealed that mean bacterial numbers and the extent of clinical signs were reduced following only two s.c. vaccinations with live *S. uberis*. However, the level of protection achieved (three out of eight quarters) was not as effective as had been attained in Group 1 (five out of six quarters). Whilst there was only a slight reduction in the milk yield of vaccinated animals in Group 3, mean number of somatic cells was raised beyond 10^4 cells ml⁻¹ milk, this level is unacceptable to the UK dairy industry¹⁰, and further supports the microbiological and clinical data which suggest that the local administration of cell wall extract does make a significant contribution to the protective effects obtained.

Previous attempts to vaccinate ruminants against mastitis-causing pathogens have been targeted towards the elevation of specific antibody in the milk and the rapid mobilization of neutrophils into the mammary gland in response to infection⁵⁻⁷. The timing and route of administration of the live *S. uberis* and the cell wall extract was therefore based upon the protocols used to generate high levels of rotavirus-specific antibody in colostrum¹¹. As in the previous study using this vaccine regime⁸, *S. uberis*-specific IgG1 was elevated in the serum of vaccinated cows in all three groups and there was little difference in the levels of *S. uberis*-specific antibody in milk, despite the omission of the intramammary booster in Group 3. This suggests that the key virulence determinants for *S. uberis* may not be somatic antigens presented on the bacterial capsule or cell wall, but could be factors produced or secreted by the live bacteria during growth *in vivo*. The production of such factors by *S. uberis* and their potential as vaccine antigens is the subject of current investigations.

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