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Published in:
Microbiology (Reading, England)

Microbiology (2005), **151**, 1369–1379 DOI 10.1099/mic.0.27788-0

CAMP factor homologues in *Propionibacterium acnes*: a new protein family differentially expressed by types I and II

Susanna Valanne,[1]† Andrew McDowell,[1] Gordon Ramage,[1]‡ Michael M. Tunney,[1]§ Gisli G. Einarsson,[1] Seamus O'Hagan,[1]‖ G. Brian Wisdom,[2] Derek Fairley,[3] Ajay Bhatia,[4] Jean-Francois Maisonneuve,[4] Michael Lodes,[4] David H. Persing[4] and Sheila Patrick[1]

Correspondence
Sheila Patrick
s.patrick@qub.ac.uk

[1]Department of Microbiology and Immunobiology, School of Medicine, Queen's University, Grosvenor Road, Belfast BT12 6BN, UK

[2]School of Biology and Biochemistry, Medical Biology Centre, 97 Lisburn Road, Queen's University, Belfast BT9 7BL, UK

[3]QUESTOR Centre, Queen's University, David Keir Building, Stranmillis Road, Belfast BT9 5AG, UK

[4]Corixa Corporation, Infectious Disease Institute, Seattle, WA 98104, USA

Analysis of the draft genome sequence of the opportunistic pathogen *Propionibacterium acnes* type strain NCTC 737 (=ATCC 6919) revealed five genes with sequence identity to the co-haemolytic Christie–Atkins–Munch-Peterson (CAMP) factor of *Streptococcus agalactiae*. The predicted molecular masses for the expressed proteins ranged from 28 to 30 kDa. The genes were present in each of the three recently identified *recA*-based phylogenetic groupings of *P. acnes* (IA, IB and II), as assessed by PCR amplification. Conserved differences in CAMP factor gene sequences between these three groups were also consistent with their previous phylogenetic designations. All type IA, IB and II isolates were positive for the co-haemolytic reaction on sheep blood agar. Immunoblotting and silver staining of SDS-PAGE gels, however, revealed differential protein expression of CAMP factors amongst the different groups. Type IB and II isolates produced an abundance of CAMP factor 1, detectable by specific antibody labelling and silver staining of SDS-PAGE gels. In contrast, abundant CAMP factor production was lacking in type IA isolates, although larger amounts of CAMP factor 2 were detectable by immunoblotting compared with type II isolates. While the potential role of the abundant CAMP factor 1 in host colonization or virulence remains to be determined, it should be noted that the type strain of *P. acnes* used in much of the published literature is a type IA isolate and is, therefore, lacking in this attribute.

Received 25 November 2004
Revised 11 February 2005
Accepted 16 February 2005

†Present address: Institute of Medical Technology, University of Tampere, Finland.

‡Present address: Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow, UK.

§Present address: School of Pharmacy, Queen's University, Belfast, UK.

‖Present address: Withers Orthopaedic Unit, Musgrave Park Hospital, Belfast, UK.

Abbreviations: CAMP, Christie–Atkins–Munch-Peterson; IFM, immunofluorescence microscopy.

The GenBank/EMBL/DDBJ accession numbers for the CAMP factor sequences of *P. acnes* strains NCTC 737 (IA), KPA171202 (IB), NCTC 10390 (II) and SG2 (II) are given in Table 3.

INTRODUCTION

As a member of the resident human microbiota, the Gram-positive anaerobic coryneform bacterium *Propionibacterium acnes* is found predominantly in the sebaceous gland-rich areas of the skin in adults (Eady & Ingham, 1994). It can, however, also be isolated from the conjunctiva, the external ear canal, the mouth, the upper respiratory tract and, in some individuals, the intestine (Funke *et al.*, 1997). It accounts for approximately half of the total skin microbiota (Tancrede, 1992), with an estimated density of 10^2 to $10^{5–6}$ cm^{-2} (Leyden *et al.*, 1998; McGinley *et al.*, 1978). In most people it outnumbers coagulase-negative *Staphylococcus* spp. (CoNS) on the skin by 10- to 100-fold (Eady

& Ingham, 1994). *P. acnes* is a well-recognized opportunistic pathogen, especially in relation to medical implants such as central nervous system shunts (Brook & Frazier, 1991), silicone implants (Ahn *et al.*, 1996) and prosthetic hip joints, where it is recovered as frequently as CoNS (Tunney *et al.*, 1998, 1999b). It is also responsible for ocular and periocular infections and endophthalmitis (Aldave *et al.*, 1999; Clark *et al.*, 1999) and has been implicated in periodontal and dental infections (Le Goff *et al.*, 1997). Indeed, dental probing and treatment can lead to the dissemination of *P. acnes* in the bloodstream (Debelian *et al.*, 1992), which is a recognized cause of endocarditis in relation to damaged or prosthetic heart valves. *P. acnes* is also considered to play a crucial role in inflammatory acne (Eady & Ingham, 1994), since antimicrobial therapy directed against *P. acnes* results in improvement, while the development of antibiotic resistance in *P. acnes* is associated with relapse (Leyden *et al.*, 1998). The common form of acne, known as acne vulgaris, affects up to 80 % of the population at some time in their lives, making it the most common skin infection. There is also a strong association between severe forms of acne and joint pain, inflammation of the bone (osteitis) and arthritis. In patients suffering from this condition, known as SAPHO (synovitis, acne, pustulosis, hyperostosis and osteitis) syndrome, isolates of *P. acnes* have been recovered from bone biopsy samples, as well as synovial fluid and tissue (Schaeverbeke *et al.*, 1998). Immunologists have long recognized and exploited the potent adjuvant activity of *P. acnes* and utilized it in models of inflammation, although many continue to refer to it as '*Corynebacterium parvum*' (Tasaka *et al.*, 1996).

Studies by Johnson & Cummins (1972) first revealed two distinct phenotypes of *P. acnes*, known as types I and II, based on serological agglutination tests and cell-wall sugar analysis. Recently, *recA*-based sequence analysis has revealed that *P. acnes* types I and II represent phylogenetically distinct groups (McDowell *et al.*, 2005). Furthermore, a small subgroup of phylogenetically distinct type I strains with atypical mAb labelling characteristics, which we now designate type IB to distinguish them from other type I strains, designated type IA, were also described. The observation that the phenotypic differences between strains of the various *P. acnes* types reflect deeper differences in their phylogeny raises the possibility that they may also display variation in their expression of putative virulence factors.

P. acnes produces a co-haemolytic reaction with both sheep and human erythrocytes (Choudhury, 1978) similar to the Christie–Atkins–Munch-Petersen (CAMP) reaction first demonstrated in 1944 (Christie *et al.*, 1944). The CAMP reaction describes the synergistic haemolysis of sheep erythrocytes by the CAMP factor from *Streptococcus agalactiae* and the β-toxin (sphingomyelinase C) from *Staphylococcus aureus*, with the CAMP factor demonstrating non-enzymic affinity for ceramide (Bernheimer *et al.*, 1979). Examination of sphingomyelinase-treated sheep erythrocytes has revealed the formation of discrete membrane pores by recombinant *Streptococcus agalactiae* CAMP factor (Lang & Palmer, 2003). In addition to the extensive study of the CAMP factor of *Streptococcus agalactiae* (Bernheimer *et al.*, 1979; Brown *et al.*, 1974; Jurgens *et al.*, 1985, 1987; Ruhlmann *et al.*, 1988; Skalka *et al.*, 1980), a number of other Gram-positive and Gram-negative bacteria are known to produce a positive CAMP reaction, including *Pasteurella haemolytica* (Fraser, 1962), *Aeromonas* species (Figura & Guglielmetti, 1987), some *Vibrio* species (Kohler, 1988) and group G streptococci (Soedermanto & Lammler, 1996). Some of these species can also use phospholipase C (α-toxin) from *Clostridium perfringens* or phospholipase D from *Corynebacterium pseudotuberculosis* as a co-factor for haemolysis in addition to the *Staphylococcus aureus* β-toxin (Frey *et al.*, 1989). The CAMP factor genes of *Actinobacillus pleuropneumoniae* and *Streptococcus uberis* have also been identified, cloned and expressed in *Escherichia coli* (Frey *et al.*, 1989; Jiang *et al.*, 1996).

The precise role of the CAMP molecule in bacterial virulence remains unclear. It is likely that the co-haemolytic reaction represents a laboratory phenotype, or epiphenomenon, that is convenient for CAMP factor detection, but which may not be directly related to the role of the molecule in colonization and pathogenesis. The CAMP factor from *Streptococcus agalactiae* binds to the Fc region of IgG and IgM molecules, similar to the binding of IgG by *Staphylococcus aureus* protein A (Jurgens *et al.*, 1987), and partial amino acid sequence similarity between the CAMP factor protein of *Streptococcus agalactiae* and *Staphylococcus aureus* protein A has been demonstrated (Ruhlmann *et al.*, 1988). We now present evidence of differences amongst *P. acnes* types IA, IB and II in the expression of proteins with sequence similarity to the CAMP co-haemolysin.

METHODS

Bacterial isolates, media and culture conditions. The following reference strains were from the National Collection of Type Cultures (NCTC, Colindale, UK), the American Type Culture Collection (ATCC, Manassas, VA, USA) and the National Collections of Industrial, Marine and Food Bacteria (NCIMB, Aberdeen, UK): *P. acnes* NCTC 737 (=ATCC 6919) and NCTC 10390, *Propionibacterium granulosum* NCTC 10387, *Staphylococcus aureus* ATCC 25923, *Actinomyces israelii* NCTC 8047, *Actinomyces naeslundii* NCTC 10301, *Micrococcus luteus* NCIMB 13267 (formerly Fleming strain 2665) and *Bacteroides fragilis* NCTC 9343. A total of 112 isolates of *P. acnes* were examined. Sixty-seven isolates were recovered from failed prosthetic hip joints and associated bone, tissue and skin samples removed from patients attending Musgrave Park Orthopaedic Hospital, Belfast, as detailed previously (Tunney *et al.*, 1998, 1999b). In addition, a further group of *P. acnes* isolates recovered from tissue samples removed during revision arthroplasty in Sweden ($n=18$), as well as acne ($n=19$) and dental ($n=8$) infections, were kind gifts. Isolates of *Propionibacterium acidipropionici*, *Streptococcus agalactiae*, *Staphylococcus epidermidis*, *Streptococcus lactis*, *Streptococcus equi*, *Streptococcus sanguis*, *Streptococcus salivarius*, *E. coli* DH5α and *Propionibacterium avidum* were obtained from the Department of Microbiology and Immunobiology culture collection, Queen's University, Belfast.

Bacterial culture. All anaerobic strains were grown on anaerobic blood agar (ABA) (CM0972; Oxoid) or in brain heart infusion (BHI) (CM225; Oxoid) broth. Cultures were incubated at 37 °C in an anaerobic cabinet (MACS MG 1000; Don Whitley Scientific), in an atmosphere of 80 % N_2, 10 % CO_2 and 10 % H_2. All *Staphylococcus* and *Streptococcus* strains were also grown at 37 °C on blood agar (BA). *E. coli* DH5α was grown aerobically on Luria–Bertani agar plates at 37 °C. Isolates of *P. acnes* were routinely identified using the API 20A multitest identification system (bioMérieux) in accordance with the manufacturer's instructions.

Co-haemolysis assay. Co-haemolytic activity was monitored by a modification of the classical co-haemolysis reaction on sheep BA plates as originally described (Christie *et al.*, 1944). Briefly, the *Staphylococcus aureus* strain ATCC 25923 was streaked vertically onto sheep BA and the test strain was then streaked horizontally outwards from either side, starting close to, but not touching, the *Staphylococcus aureus* streak. Plates were incubated anaerobically at 37 °C for 48 h. A butterfly-shaped zone of lysis at the junction of the streaks was caused by the co-effect of diffusing *Staphylococcus aureus* β-toxin and co-haemolytic factor.

Production of mAb and rabbit polyclonal antisera. The mAb QUBPa4 was generated using the protocol described previously (Harlow & Lane, 1988; Tunney *et al.*, 1999a). Four BALB/c mice were immunized with killed whole cells (10^8 c.f.u. ml^{-1}) of *P. acnes*. The hybridoma cell line producing QUBPa4 was then cloned by limiting dilution (Harlow & Lane, 1988).

Rabbit polyclonal antisera were prepared against the five CAMP proteins using recombinant products expressed in *E. coli*. CAMP genes were amplified from *P. acnes* NCTC 737 genomic DNA and subcloned into the plasmid vector pET17b (Stratagene). Ligation products were first transformed into *E. coli* XL-1 Blue competent cells (Stratagene) and the plasmid DNA isolated from XL-1 Blue transformants was subsequently transformed into *E. coli* BL21 (DE3) pLysE or pLysS host cells (Novagen). The recombinant proteins were expressed in *E. coli* with a poly-histidine tag at the N terminus and were purified from IPTG-induced batch cultures, in the presence of 8 M urea, by affinity chromatography using the one-step QIAexpress Ni-NTA agarose matrix (Qiagen). Purity of the recombinant proteins was assessed by SDS-PAGE, followed by Coomassie brilliant blue staining and N-terminal sequencing using Edman chemistry with a Procise 494 protein sequencer (Perkin-Elmer Applied Biosystems). All recombinant proteins were assayed for endotoxin contamination using the *Limulus* amoebocyte assay (BioWhittaker) and shown to contain less than 50 endotoxin units mg^{-1}. Polyclonal rabbit antiserum was raised against all recombinant proteins by injecting New Zealand white rabbits (R&R rabbitry) with 200 μg purified recombinant protein in incomplete Freund's adjuvant (IFA) plus 100 μg muramyl dipeptide (Sigma). Serum was collected following two subsequent boosts separated by 3 weeks with 100 μg protein in IFA. As a result of problems with stability of the CAMP factor 1 and 5 recombinant proteins, only an N-terminal fragment of the CAMP 1 protein and N-terminal and C-terminal fragments of the CAMP 5 protein were used for immunization.

Patient serum. Serum was prepared from venous blood (10 ml) taken pre-operatively from patients about to undergo either primary or revision total hip arthroplasty at Musgrave Park Hospital, Belfast, and from acne patients attending a Dermatology outpatients clinic in the Royal Victoria Hospital, Belfast. These procedures were approved by the local ethical committee and all patients gave full consent.

Immunofluorescence microscopy (IFM). IFM was carried out as described previously (Patrick *et al.*, 1995) with minor modification. Briefly, bacterial cultures were grown on ABA or BA and a suspension of 10^8 c.f.u. ml^{-1} was prepared in 0·01 M PBS (0·15 M NaCl, 0·0075 M Na_2HPO_4, 0·0025 M $NaH_2PO_4.2H_2O$; pH 7·4). Samples (10 μl) were then applied to multiwell slides, air-dried and fixed in 100 % methanol for 10 min at −20 °C. Undiluted hybridoma cell culture supernatant containing mAb QUBPa4 (30 μl) was added to each well of the slides and incubated for 45 min at 37 °C. After washing in 0·01 M PBS for 20 min at room temperature, a 1 : 100 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma) in PBS containing 0·1 % (w/v) Evans Blue (Merck Sharp & Dome) counterstain (30 μl) was applied to each well and incubated for 45 min at 37 °C. Control wells in which primary antibody was replaced with PBS were routinely included to monitor non-specific binding of the secondary antibody. Slides were then washed and mounted in glycerol-PBS, containing an anti-photobleaching agent (Citifluor; Agar Scientific), and examined using a Leitz Dialux 20 fluorescence microscope.

Preparation of bacterial extracts. To obtain efficient extraction and reproducible recovery of loosely cell-associated and secreted CAMP factor 1, standardized whole cell bacterial preparations of 1×10^{10} c.f.u. ml^{-1} were obtained by suspending a culture grown for 6 days on ABA directly into PBS, such that a 1 : 100 dilution had an OD_{600} of 0·3. Cells were then disrupted by ultrasound (Soniprep 150; 26 μm amplitude) for 5 min at 4 °C. The sonicated suspension was brought to room temperature and Tween 20 (Bio-Rad) was added to a final concentration of 2 mM. The sample was then centrifuged and the resulting pellet discarded. Sodium azide was added to the supernatant (final concentration 0·02 % v/v) before storage at −20 °C.

To investigate the presence of secreted CAMP factor 1 protein in culture supernatant, bacteria were grown in BHI broth for 24 h and then centrifuged at 2370 ***g*** for 30 min (Mistral; MSE) at room temperature and the supernatant retained. An equal volume of ice-cold ethanol was then added to the supernatant followed by overnight incubation at 4 °C. The precipitated material was recovered by centrifugation at 12 000 ***g*** for 30 min (Sorvall) and the pellet was resuspended in 500 μl distilled water.

SDS-PAGE and immunoblotting. Bacterial extracts, prepared as detailed above, were analysed using 9 % SDS-PAGE gels (Laemmli, 1970) and the resolved proteins were visualized using a silver staining kit (Amersham Pharmacia Biotech). To afford standardization and comparison of different *P. acnes* isolates for CAMP factor 1 expression, the colour development was carried out for between 110 and 120 s. Gels were washed three times for 5 min each in distilled water, placed in a drying solution [30 % (v/v) ethanol, 5·3 % (v/v) glycerol] for two periods of 30 min and preserved between cellophane sheets. The gels were then photographed using a Kodak DC290 digital camera fitted on a Kodak EDAS290 gel imaging hood and images were analysed using Kodak IK image analysis software version 3.5. Known positive and negative strains were included in each experiment as internal controls and indicated that the experimental system was reproducible.

Immunoblotting was carried out as described previously with a minor modification (Patrick & Lutton, 1990). In brief, nitrocellulose was blocked with 0·01 M PBS containing 0·05 % (v/v) Tween 20 (PBST) and 5 % (w/v) non-fat milk powder (Marvel; Premier brands). After washing with PBST, the nitrocellulose was incubated in undiluted mAb supernatant or an appropriate dilution of polyclonal antiserum in PBS. The nitrocellulose was then washed in PBST before incubation with alkaline phosphatase-conjugated goat anti-human IgG (H), anti-mouse IgG (H + L) or anti-rabbit IgG (H + L) (Sigma). Controls in which the primary antibody was replaced by PBS were routinely included to monitor non-specific binding of the secondary antibody.

Bound antibodies were detected using an alkaline phosphatase conjugate substrate kit containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

Purification and analysis of CAMP factor 1 protein. The mAb QUBPa4 was purified using a HiTrap protein G column (Amersham Biosciences), concentrated by ultrafiltration and immobilized on CNBr-activated agarose (Sigma). The antibody gel was packed into a column and equilibrated with PBS. Extracts of ABA-grown *P. acnes* (isolate SG2), prepared as detailed above, were diluted in PBS and recycled through the column overnight. The bound antigen was released with 100 mM glycine/HCl buffer, pH 2·7, and immediately neutralized with 1 M Tris before storage at −20 °C. For trypsin digestion, the eluted fractions from the affinity chromatography column, containing approximately 120 μg purified protein, were pooled and dialysed against distilled water at 4 °C overnight. The sample was then lyophilized using a Speed-Vac (Savant), the precipitate dissolved in 20 μl 8 M urea, 0·4 M NH_4CO_3 and the pH adjusted to between 7·5 and 8. DTT (0·25 μmol) was added and the suspension was incubated at 50 °C for 15 min. After cooling to room temperature, iodoacetic acid (0·5 μmol) was added and the suspension was incubated at room temperature for a further 15 min. The final concentration of urea was then reduced to 2 M (final volume of digest 80 μl) by the addition of 50 μl distilled water. Trypsin (2·5 mg ml^{-1}; Roche) was added in a 1 : 25 ratio to the CAMP factor 1 protein (w/w) and the mixture was incubated at 37 °C for 24 h. Digestion was stopped by freezing the sample at −20 °C. Purified antigen and its fragments were subjected to SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad). The bands were stained with Coomassie brilliant blue, excised and sequenced by Edman chemistry at the Babraham Institute (Cambridge, UK).

PCR amplification and sequencing. PCR was used to detect the five CAMP factor homologue genes in a selection of *P. acnes* strains. CAMP factor genes were amplified using primers directed to downstream and upstream flanking sequences of each ORF (based on the *P. acnes* NCTC 737 genome sequence), thus facilitating accurate sequence determination of the 5′ and 3′ ends of each ORF. Preparation of bacterial genomic DNA and PCR amplifications were carried out essentially as described previously (McDowell *et al.*, 2005). PCR samples contained 1 × PCR buffer, 200 μM of each dNTP (Amersham Pharmacia Biotech), 200 μM of each appropriate CAMP factor oligonucleotide primer (Table 1), 1·5 mM $MgCl_2$, 1·25 U Platinum *Taq* DNA polymerase (Invitrogen Life Technologies) and 2·5 μl bacterial lysate, in a total volume of 25 μl. Samples were initially heated at 95 °C for 3 min, followed by 35 cycles of 1 min at 95 °C, 30 s at the appropriate annealing temperature (Table 1) and 1 min at 72 °C. The PCR was completed with a final extension step at 72 °C for 10 min. A negative water control was included in all experiments. All PCR products were analysed as described before (McDowell *et al.*, 2005). Sequencing reactions were performed using ABI PRISM Ready Reaction Terminator cycle sequencing kits (Perkin Elmer Applied Biosystems) according to the manufacturer's instructions. Samples were analysed on an ABI PRISM 3100 DNA sequencer (Perkin Elmer Applied Biosystems).

Sequence and phylogenetic analyses. A draft sequence of the *P. acnes* NCTC 737 genome was commissioned from Genset (Evry, France) by Corixa Corporation (Seattle, WA, USA). A total of 26 million bases of DNA were sequenced, representing approximately 10 genome equivalents. An annotated database of ORFs was created using seven Genemark predictive models, as well as BLASTP, Psort, SignalP, Pfamm, InterProScan and other bioinformatic applications. Comparison with the *P. acnes* KPA171202 genome sequence (Bruggemann *et al.*, 2004) was carried out using the Artemis Comparison Tool (ACT; http://www.sanger.ac.uk/Software/ACT/). Consensus trees showing protein and nucleotide sequence relationships were generated using the Data Analysis in Molecular Biology and Evolution software (DAMBE; http://aix1.uottawa.ca/~xxia/software/software.htm). Multiple sequence alignments were performed using the CLUSTAL W algorithm (Thompson *et al.*, 1994) and exported into the DAMBE program. For nucleotide analysis, consensus trees were constructed using the maximum-parsimony method and the neighbour-joining method using the Jukes–Cantor-based algorithm. Sequence input order was randomized and bootstrapping resampling statistics were performed using 100 datasets for each analysis.

RESULTS

Identification of multiple genes with CAMP factor sequence identity

Analysis of the draft genome sequence of the *P. acnes* type IA strain NCTC 737 (=ATCC 6919) (property of Corixa Corporation) identified five related genes, sited at different locations within the genome, with similarity to the *Streptococcus agalactiae* co-haemolysin or CAMP factor protein (GenBank accession no. X72754.1) originally described by Christie, Atkins and Munch-Petersen in 1944 (Christie *et al.*, 1944). The resulting CAMP factor protein sequences had predicted molecular masses that ranged from approximately 28 to 30 kDa. All five genes have a putative N-terminal signal peptide cleavage site (Table 2).

Table 1. PCR primers used in this study for amplification of *P. acnes* CAMP factor genes

Target	Primer	Sequence (5′–3′)	Position	Annealing temperature (°C)	Product size (bp)
CAMP 1	C1-F	GCTTGCAGTTGCGAGCAATTGTTC	−44 to −21	60	946
	C1-R	CCCATGCCGTAAATGATTTCGATG	902 to 879		
CAMP 2	C2-F	GTCGTAGCCATACACCACACG	−172 to −152	60	1015
	C2-R	GCACCGAGTGTTGATGTCAATTAGC	843 to 819		
CAMP 3	C3-F	AATCGTGGCGGGGAGGTTAGTA	−102 to −81	62	1000
	C3-R	GACACGTCAATAGGGGAGAAGAAG	898 to 875		
CAMP 4	C4-F	CAGATCGACCAACGCTTTAGG	−139 to −119	58	1039
	C4-R	CCACCACAGGCACGGATTGA	900 to 881		
CAMP 5	C5-F	CCACGCCATGAGCTAAGGACAG	−246 to −225	62	1178
	C5-R	TGAACTAGACCGCGGCAAACATT	932 to 910		

Table 2. Characteristics of the predicted *P. acnes* CAMP factor proteins of NCTC 737, KPA 171202 and NCTC 10390

Strains belong to types IA (NCTC 737), IB (KPA171202) and II (NCTC 10390).

Protein	Calculated molecular mass (Da)*			Amino acids	Theoretical pI			Putative signal sequence cleavage site (positions)
	NCTC 737	KPA 171202	NCTC 10390		NCTC 737	KPA 171202	NCTC 10390	
CAMP 1	30 391	30 376	30 160	285	9·77	9·88	9·74	AHA–AP (27–28)
CAMP 2	28 538	28 608	28 673	267	9·52	9·61	9·52	AHA–VE (27–28)
CAMP 3	29 042	29 210	29 159	271	9·50	9·67	9·59	AVA–AP (25–26)
CAMP 4	28 245	28 211	28 273	267	9·50	9·50	9·50	AQA–SA (27–28)
CAMP 5	29 855	29 821	29 905	281	9·68	9·68	9·61	AQA–AX† (27–28)

*Including signal sequence.
†V in type I; I in type II.

Construction of a bootstrapping consensus tree (using 100 bootstrapping replications) based on the different *P. acnes* CAMP factor protein sequences of NCTC 737, along with the CAMP factor sequences from *Streptococcus agalactiae* (GenBank accession no. NP_736433.1), *Streptococcus uberis* (accession no. AAA78910.1) and *Streptococcus pyogenes* (accession no. NP_802366.1), revealed that CAMP factor 3 was more distantly related to the other *P. acnes* CAMP factor proteins (Fig. 1). The *P. acnes* CAMP factors 1 and 5 were found to be more closely related to one another, as were CAMP factors 2 and 4.

Nucleotide sequence analysis of CAMP factor genes in *P. acnes* types I and II

The presence of all five CAMP factor genes in other strains of *P. acnes* type IA, as well as strains of type IB and type II, was revealed by PCR with primers designed to downstream and upstream flanking sequences of each CAMP factor ORF. PCR analysis of 12 isolates representative of *P. acnes* type IA (NCTC 737, RM1, PV37), type IB (CK17, LED2, RM9, W1392, W1998) and type II (NCTC 10390, SG2, RM4 and P24) resulted in PCR products of the correct size for all five CAMP factor genes, in all the isolates examined (data not shown). Nucleotide sequence analysis of all five CAMP factor genes from strains selected to represent type IA (NCTC 737) and type II (NCTC 10390 and SG2) was carried out (Table 3). These sequences were then compared with the CAMP factor gene sequences from the recently published genome sequence of *P. acnes* KPA171202 (Bruggemann *et al.*, 2004; accession no. AE017283), which we identified as a type IB strain based on *recA* and *tly* phylogenetic analysis. Consensus trees of the individual CAMP factor gene sequences from these strains were constructed using the maximum-parsimony and neighbour-joining methods. After 100 bootstrapping replications, the consensus trees derived using the two methods gave identical topologies. For CAMP factors 1, 2, 4 and 5, the phylogenetic relationships between the IA, IB and II strains were identical to that determined previously by *recA* and *tly* gene analysis (illustrated in Fig. 2a for CAMP factor 1). For CAMP 3, however, the consensus tree differed, as the type IA sequence was found to have a closer relationship to the type II sequence, with the type IB sequence being more distinct (Fig. 2b).

Comparison was also made between strains NCTC 737 (IA) and KPA171202 (IB) for nucleotide and amino acid

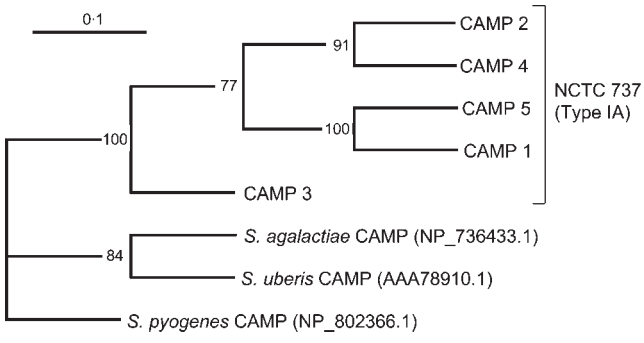

Fig. 1. Consensus tree illustrating the relationship between CAMP factor protein sequences from *P. acnes* NCTC 737 (type IA), *Streptococcus agalactiae*, *Streptococcus uberis* and *Streptococcus pyogenes*. Multiple sequence alignments were performed and the resulting phylogenetic tree was rooted with the *Streptococcus pyogenes* sequence. Bootstrapping resampling statistics were applied to the trees (100 datasets) and bootstrap values are shown at each node of the tree.

Table 3. GenBank accession numbers for CAMP factor sequences of *P. acnes* NCTC 737 (IA), KPA171202 (IB), NCTC 10390 (II) and SG2 (II)

Protein	NCTC 737	KPA171202	NCTC 10390	SG2
CAMP 1	AY527218	AAT83089	AY787764	AY787769
CAMP 2	AY726656	AAT82444	AY787765	AY787770
CAMP 3	AY726657	AAT83815	AY787766	AY787771
CAMP 4	AY726658	AAT82980	AY787767	AY787772
CAMP 5	AY726659	AAT82947	AY787768	AY787773

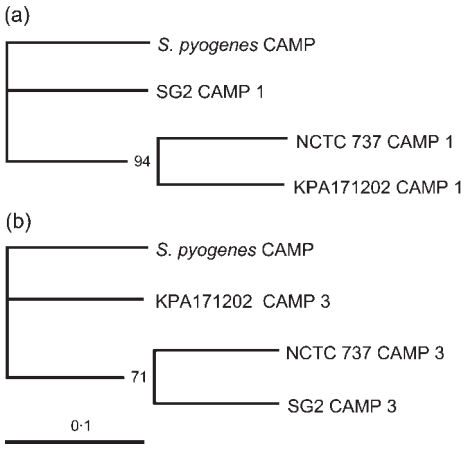

Fig. 2. Consensus trees illustrating the relationship between *P. acnes* types IA, IB and II CAMP factor genes. Multiple alignments of CAMP factor 1 (a) and CAMP factor 3 (b) gene sequences from strains NCTC 737 (type IA), KPA171202 (type IB) and SG2 (type II) were performed, along with the published CAMP sequence of *Streptococcus pyogenes*, which was used as an outgroup for the phylogenetic analysis. Consensus trees were constructed using the neighbour-joining method (with Jukes–Cantor-based algorithm). Bootstrapping resampling statistics were applied to the trees (100 datasets) and bootstrap values are shown at each node on the trees.

sequence differences in proteins putatively involved in the degradation of host molecules, as well as those thought to be cell-surface-associated. Analysis revealed variable numbers of nucleotide and amino acid sequence differences between the two strains (Table 4).

CAMP factor homologues of *P. acnes* and IgG-binding domains of protein A

As IgG binding by the *Streptococcus agalactiae* CAMP factor (Jurgens *et al.*, 1987), as well as partial amino acid sequence similarity of the molecule to protein A of *Staphylococcus aureus* (Ruhlmann *et al.*, 1988), has been demonstrated, sequence comparisons were made between the *P. acnes* CAMP factors and staphylococcal protein A. CLUSTAL W analyses identified a protein domain between positions 108 and 157 in CAMP factor 1 with 25 % or more identity to the IgG-binding domains E, B and C of *Staphylococcus aureus* protein A (Table 5). In addition, CAMP factor 3 showed approximately 25 % identity to domain A between positions 104 and 142. The CAMP factor of *Streptococcus agalactiae* was found to have considerably lower percentage identity.

Co-haemolytic (CAMP) reactivity of *P. acnes* types I and II

Fifty isolates of *P. acnes* (types IA, IB and II) recovered from failed prosthetic hip joints and associated bone and tissue samples, as well as skin and dental sources and strains of *Staphylococcus epidermidis*, *A. israelii*, *A. naeslundii* and *Streptococcus agalactiae*, were positive for the co-haemolytic CAMP reaction on sheep BA using a positive β-toxin-producing strain of *Staphylococcus aureus* (ATCC 25923). All *P. granulosum* strains were negative for the CAMP reaction, as were strains of *P. avidum*, *P. acidipropionici*,

Table 4. Percentage identity and number of nucleotide/amino acid differences between KPA171202 (type IB) and NCTC 737 (type IA) CAMP factors, selected putative host cell molecule-degrading factors and antigens

KPA171202 ORF	Putative function	Nucleotide sequence		Amino acid sequence	
		Differences (*n*)	Identity (%)	Differences (*n*)	Identity (%)
0687	CAMP factor	1	99·9	1	99·6
1198	CAMP factor	1	99·9	1	99·6
1231	CAMP factor	1	99·9	1	99·6
1340	CAMP factor	2	99·8	2	99·3
2108	CAMP factor	9	98·9	3	98·5
1396	Haemolysin	2	99·7	1	99·6
0685	Sialidase	2	99·9	0	100
1560	Sialidase	39	97·2	18	96·3
1569	Sialidase	56	98·6	19	98·6
1796	Triacyl glycerol lipase	37	96·4	11	96·5
2105	Triacyl glycerol lipase	9	99·1	6	98·2
1745	Phosphoesterase	3	99·9	2	99·8
0109	Myosin cross-reactive antigen	47	97·4	7	98·8
0453	GroEL	10	99·4	0	100
1955	Surface protein	57	96·9	27	95·6

Table 5. Comparison of predicted CAMP factor amino acid sequences of *P. acnes* type IA (NCTC 737) and type II (NCTC 10390) with the IgG-binding domains of *Staphylococcus aureus* protein A

Values are percentages of identity. Where type IA and II differ, the values are listed before (NCTC 737) and after (NCTC 10390) the solidus (/).

CAMP factor	*Staphylococcus aureus* protein A IgG-binding domain				
	E	D	A	B	C
1	25·0	23·2	17·6	28·0	30·0
2	12·0/14·0	14·9/15·7	15·7	10·9/15·7	13·5
3	19·2	19·3	25·6	19·1	17·0
4	15·4	16·7	14·5	13·0	15·7
5	18·4	11·1	14·0	15·8	22·0
Streptococcus agalactiae	12·2	11·8	11·5	13·5	15·4
Streptococcus uberis	14·3	18·0	17·5	14·8	16·7
Streptococcus pyogenes	15·4	14·8	19·6	17·6	17·6

M. luteus, *Streptococcus equi*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Streptococcus lactis*, *E. coli* DH5α and *B. fragilis*.

Differences in CAMP factor protein expression between *P. acnes* types I and II

Expression of the five CAMP factor proteins by type I and II isolates was investigated by immunoblotting with rabbit polyclonal antisera raised against recombinant forms of each NCTC 737 CAMP factor protein, as well as a mouse mAb specific for CAMP factor 1. Representative immunoblots of NCTC 737 (type IA) and the prosthetic hip joint isolate SG2 (type II) are presented in Fig. 3. Labelled bands were observed in the correct region for the predicted molecular mass of the proteins, minus the signal sequence. Bands of lower molecular mass than that of the predicted secreted protein were sometimes observed and are believed to relate to degradation of a predicted labile N-terminal region. Isolates of *P. acnes* type II were found to produce large amounts of CAMP factor 1 compared with type IA strains. A corresponding abundant protein band was also observed on silver-stained SDS-PAGE gels of *P. acnes* type II, but was absent in type IA isolates (Fig. 4) and NCTC 10390 (type II; data not shown). Larger quantities of CAMP 2, however, were detectable in type IA isolates compared with type II by immunoblotting, although levels of the protein were still considerably less than CAMP factor 1 production (Fig. 3). Analysis of strains of *P. acnes* type IB by immunoblotting and silver staining of SDS-PAGE gels revealed a pattern of CAMP factor expression similar to that of type II organisms, with the production of large amounts of CAMP factor 1 and reduced expression of CAMP factor 2 compared with type IA strains (data not shown). Nucleotide sequences immediately upstream of the CAMP genes were compared to determine whether sequence differences in these regions could be influencing expression. Analysis revealed conservation of putative core Shine–Dalgarno sequences amongst the different *P. acnes* types for CAMP factors 1, 2, 3 and 5, but one base difference in the CAMP factor 1 Shine–Dalgarno sequence of NCTC 10390 was observed. The upstream sequences of CAMP factor 4 were more variable. The CAMP factor 4 gene for all isolates examined had a GTG start codon, whereas the start codon for all other CAMP factors was ATG (Fig. 5).

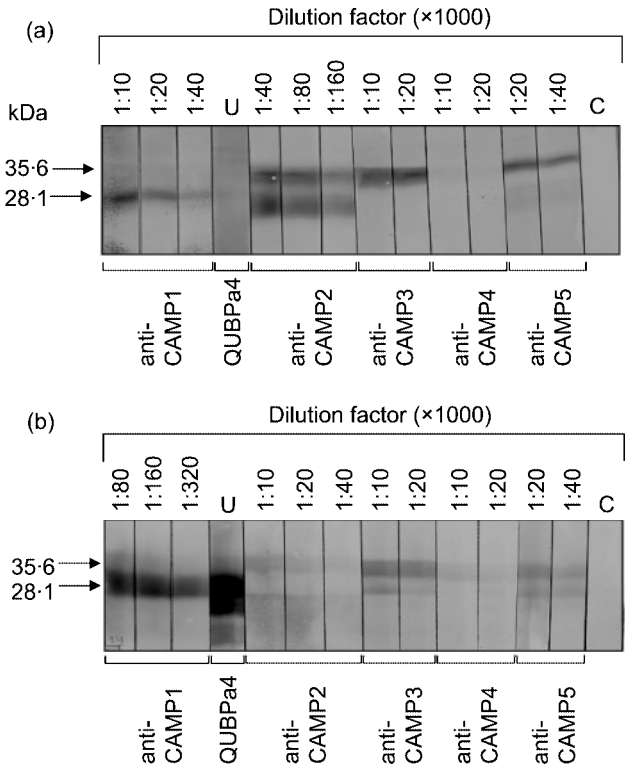

Fig. 3. Immunoblots probed with rabbit anti-CAMP factor protein polyclonal antisera and mouse anti-CAMP factor 1 mAb (QUBPa4). (a) *P. acnes* NCTC 737 (type IA); (b) *P. acnes* SG2 (type II). C, Control with no primary antibody; U, undiluted.

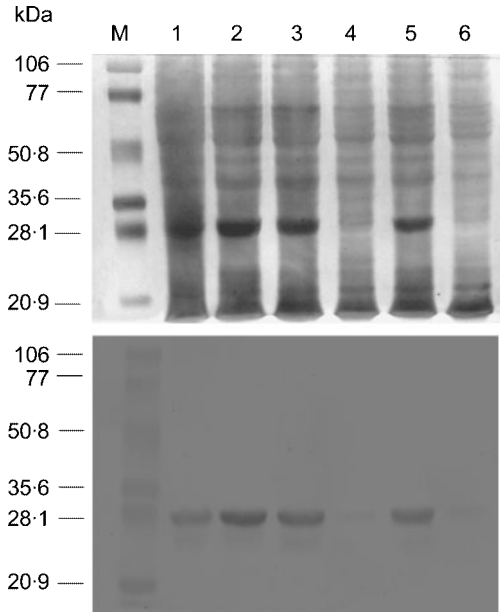

Fig. 4. Analysis of *P. acnes* extracts by SDS-PAGE followed by silver staining (top) or immunoblotting with a mouse anti-CAMP factor 1 mAb (QUBPa4) (bottom). Lanes: 1–3 and 5, type II *P. acnes* isolates JJK1 (lane 1), HMJ3 (2), SG2 (3) and PC3 (5); 4 and 6, type IA *P. acnes* isolates SB1 (4) and AT3 (6). Lanes M, molecular mass markers (Bio-Rad, low range).

CAMP 1 *

NCTC 10390 CGATGAAC**GGAA**CCCACAAATG
NCTC 737 CGATGAA**AGGAA**CCCACAAATG
SG2 CGATGAA**AGGAA**CCCACAAATG

CAMP 2
Consensus CAATAAAC**GGAG**AACCTTTATG

CAMP 3
Consensus TCCCTCGA**GGAGG**ATTCCCATG

CAMP 4
NCTC 737 CTGCGATTGT**AG**ATTCATCGTG
KPA171202 CTGCGATT**GGAG**ATTCATCGTG
SG2 CTGCGATT**GGAGG**TTCATCGTG

CAMP 5
Consensus CGAAAAAGA**GGAG**AACTCTATG

Fig. 5. Comparison of upstream regions of *P. acnes* CAMP factor genes. Consensus sequence for *P. acnes* NCTC 737 (type IA), KPA171202 (type IB) and SG2 (type II). Base matches representing equivalent DNA sequence of putative core Shine–Dalgarno sequences are illustrated in bold. *, Mismatch in NCTC 10390.

Comparison of CAMP factor 1 expression by *P. acnes* type I and II isolates

Comparison of CAMP factor 1 expression between types IA, IB and II was performed by silver staining of bacterial extracts resolved on SDS-PAGE gels. Confirmation that the abundant protein band observed was CAMP factor 1 was obtained by immunoblotting with our mAb specific for the CAMP factor 1 protein (QUBPa4; Fig. 4). Specificity of the mAb was confirmed by N-terminal sequencing of affinity-purified complete and trypsin-digested protein (data not shown). The staining intensity of the 28 kDa CAMP factor 1 band in extracts from NCTC 737 and NCTC 10390 was compared, as well as 76 type IA, 5 type IB and 31 type II isolates recovered from a variety of sources. The type IA strain NCTC 737 was used as an internal standard on all gels (arbitrary value of 1·0). All 31 type II *P. acnes* isolates, from failed prosthetic hip joints and associated bone and tissue samples, as well as one acne isolate, had a band intensity for CAMP factor 1 that was at least sixfold greater than that for NCTC 737 (mean = 14). The type II strain NCTC 10390 was an exception and lacked an intense band in this region of the gel, consistent with its absence after immunoblotting (not illustrated). The five type IB isolates recovered from dental sources ($n=2$), a failed prosthetic hip joint ($n=1$), a prosthetic hip joint-associated tissue sample ($n=1$) and a skin sample from the hip of a patient undergoing revision arthroplasty ($n=1$), also had at least sixfold greater band intensity for CAMP factor 1 (mean = 18). Type IA isolates recovered from dental sources ($n=6$), failed prosthetic hip joints and associated bone and tissue samples ($n=52$) and patients with acne ($n=18$) had a band intensity in the range of 1–9 (mean = 3).

Immunofluorescence labelling of CAMP factor 1 on whole cells of *P. acnes*

IFM with mAb QUBPa4 indicated that the CAMP factor 1 protein was both cell-associated and secreted (Fig. 6a). Mouse mAbs of the same isotype as QUBPa4, specific for cell-surface components of *P. acnes* I and II, respectively, did not label extracellular material by IFM (Fig. 6b, c). A centrifugation wash in PBS of bacteria harvested from agar plates was sufficient to remove most of the CAMP factor 1 protein (Fig. 6d, e). In addition, we also found that the protein could be obtained from broth culture after 50-fold concentration by ethanol precipitation. *P. acnes* type II strain NCTC 10390, which did not react with QUBPa4 by immunoblotting, was also non-reactive by IFM. IFM revealed no reactivity of QUBPa4 with *P. granulosum*, *P. acidipropionici*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus agalactiae* (data not shown). Although *A. israelii* and *A. naeslundii* showed no reaction with QUBPa4 by IFM, reactivity was observed after SDS-PAGE and immunoblotting (data not shown).

Reactivity with patient antisera

The sera from acne patients ($n=9$) and patients undergoing primary total hip arthroplasty ($n=10$) and revision

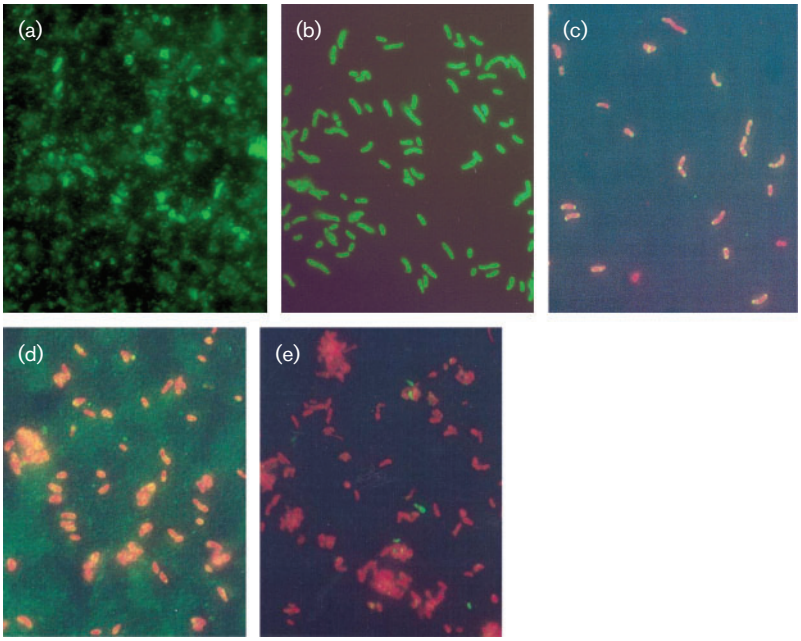

Fig. 6. Immunofluorescence micrographs of *P. acnes* clinical isolates labelled with mouse isotype IgG2b mAbs. (a)–(c) Type II isolate labelled with QUBPa4, specific for CAMP 1 (a), type II isolate labelled with QUBPa2, specific for *P. acnes* type II-associated carbohydrate antigen (b) and *P. acnes* type IA clinical isolate labelled with mouse isotype IgG2b mAb QUBPa5, specific for a protein antigen associated with type IA (c). (d)–(e) Unwashed and washed *P. acnes* type IA clinical isolate labelled with CAMP factor 1-specific QUBPa4: bacterial suspension dried and fixed directly onto slide (d) and bacterial suspension first centrifuged and then resuspended in PBS (e). Images were viewed with fluorescein-detecting filter (a, b) or with combined fluorescein- and Evans Blue-detecting filter (c–e). Microscope magnification, ×1000.

arthroplasty ($n=11$) were examined by immunoblotting at a single dilution of 1 : 500 for their reactivity against immunoaffinity-purified CAMP factor 1 protein from a type II isolate (SG2). In total, sera from three patients with acne, five patients undergoing primary arthroplasty and four patients undergoing revision arthroplasty were reactive.

DISCUSSION

A total of five individual genes with sequence identity to the co-haemolytic CAMP factor, originally described in *Streptococcus agalactiae* (Christie *et al.*, 1944), were identified in the draft genome sequence of the *P. acnes* type IA strain NCTC 737. Recently, these genes have been independently identified in the genome sequence of the *P. acnes* strain KPA171202 (Bruggemann *et al.*, 2004), which we identified as type IB based on comparison of *recA* and *tly* sequences (McDowell *et al.*, 2005). PCR analysis of a selection of type IA, IB and type II isolates indicated that all five genes are present in each of the different *P. acnes* types. To our knowledge, multiple CAMP homologues have not been described in any other organisms. The conserved nucleotide and amino acid sequence differences observed with the CAMP factors from all three *P. acnes* types provided additional evidence for the phylogenetically distinct nature of the groups and confirmed previous results obtained with *recA* and *tly* genes (McDowell *et al.*, 2005). In contrast, CAMP factor sequences from two serotypes of *Streptococcus agalactiae* (serotype III strain NEM316 and serotype V strain 2603V/R) have been shown to be identical (Glaser *et al.*, 2002). In addition, varying numbers of nucleotide and amino acid differences were observed between the sequences of NCTC 737 (1A) and KPA171202 (1B) for a selection of putative secreted and cell-surface-associated proteins, providing further evidence for their designation as distinct phylogenetic groups within type I (McDowell *et al.*, 2005).

Analysis of CAMP factor protein expression by immunoblotting and silver staining of SDS-PAGE gels revealed an abundance of CAMP factor 1 production by type II and type IB isolates, but not IA organisms. The type II strain NCTC 10390, however, was an exception, as abundant CAMP factor 1 protein was not detected. As the genes for all five CAMP factors are present in all three *P. acnes* groups, observed differences reflected different levels of expression rather than missing genes. One factor amongst many that could influence expression levels is the interaction between a Shine–Dalgarno ribosome-binding site and 16S rRNA. Strong Shine–Dalgarno sequences (e.g. GGAG, GAGG or AGGA) are associated with genes that are predicted to be highly expressed (Karlin & Mrazek, 2000). Interestingly, we identified a base difference in the putative Shine–Dalgarno sequence associated with the CAMP factor 1 gene of NCTC 10390, a type II strain that lacks abundant CAMP factor 1 production and is not reactive with our specific mAb by IFM. Whether or not this reduces the efficiency of interaction with the 16S ribosomal subunit, and therefore the level of expression of the protein, remains to be determined. The observation that NCTC 10390 differs from other type II organisms with respect to CAMP factor 1 production indicates that it is not the most appropriate representative strain of *P. acnes* type II.

Immunoblotting also revealed that type IA isolates express greater quantities of CAMP factor 2 compared with type II and type IB isolates; however, neither this nor the other CAMP factors were produced in quantity by IA strains, as the abundant protein band was not detectable by SDS-PAGE and silver staining. The putative Shine–Dalgarno

sequences of CAMP factors 1 and 2 are conserved for all three phylogenetic groups; therefore, this cannot explain the differences in expression. No striking differences were observed with respect to the expression of CAMP factor proteins 3, 4 and 5, although CAMP factor 4 reacted only weakly in immunoblotting experiments with type IA and II isolates. The putative Shine–Dalgarno sequences upstream of the CAMP factor 4 genes were more varied amongst the three phylogenetic groupings, but comparison with the Shine–Dalgarno sequences of the other CAMP genes did not reveal any clear relationship that could explain the different expression levels. CAMP factor 4 does, however, have a GTG start codon, which is reported to be a weaker translational initiator than ATG (Ringquist *et al.*, 1992).

All of the CAMP factor sequences contain a putative signal sequence cleavage site (Table 2) and molecular mass comparison of the proteins by SDS-PAGE and immunoblotting was in keeping with the loss of this signal sequence. IFM analysis and detection in ethanol-precipitated supernatant from broth culture confirmed that the CAMP factor 1 protein was secreted, although it was also detected on the surface of the *P. acnes* cells. Studies with *Streptococcus agalactiae* similarly detected CAMP factor protein in the external milieu, as well as on the cell surface (Jurgens *et al.*, 1987). None of the CAMP factor homologues from *P. acnes* or *Streptococcus agalactiae* contain a C-terminal Leu-Pro-X-Thr-Gly (LPXTG) motif, although 25 genes encoding other proteins with an LPXTG motif have been described in the genome of *P. acnes* strain KPA171202 (Bruggemann *et al.*, 2004).

Despite our observations of differential expression of the five CAMP factor homologues amongst *P. acnes* types IA, IB and II, all isolates from both groups were positive for the co-haemolytic phenotype. The *P. acnes* co-haemolytic reaction is therefore likely to be mediated by more than one CAMP factor protein. We are currently addressing the relationship between individual CAMP factors and the co-haemolytic phenotype. In our studies, some sequence identity between the *P. acnes* CAMP factor 1 and 3 proteins and *Staphylococcus aureus* protein A was demonstrated within the Fc-binding region, suggesting they may have immunoglobulin-binding activity. Interestingly, the percentage identities were greater than those of the *Streptococcus agalactiae* CAMP factor, which has been shown to bind IgG (Jurgens *et al.*, 1987). It may be that the multiple *P. acnes* CAMP factors have arisen from divergence of a replicated common ancestral gene and now have divergent functions.

In addition to the *P. acnes* isolates studied, *A. israelii* and *A. naeslundii* produced a co-haemolytic reaction whereas *P. granulosum* did not. It would be interesting to determine whether this difference reflects the relative pathogenic potential of these organisms. The positive reaction between purified CAMP factor 1 protein and human sera obtained from patients with acne, as well as those undergoing primary or revision hip arthroplasty, indicates that the protein is expressed by *P. acnes* during human colonization. Whether its production relates to virulence remains to be determined, as the serum samples used were from a single time point and therefore give no indication of rising titre.

In conclusion, we have identified five genes with sequence identity to the co-haemolytic CAMP factor of *Streptococcus agalactiae* in strains of *P. acnes* types IA, IB and II. Differential protein expression of the CAMP factors amongst the various *P. acnes* phylogenetic groupings was observed; in particular, the extracellular and cell-associated CAMP factor 1 protein was produced in striking abundance by type IB and type II isolates. The observation of differential expression of putative virulence determinants amongst the various *P. acnes* types will have important consequences. In particular, it will impact on our interpretation of previously published virulence data that have been based on the study of only one isolate type, such as NCTC 737, which has often been used as a model organism for studies of *P. acnes* virulence (Roszkowski *et al.*, 1980; Webster *et al.*, 1985). More generally, such data, in combination with the recent demonstration that *P. acnes* is genetically heterogeneous, will serve to challenge our current understanding of the virulence and pathogenic potential of clinical isolates of *P. acnes*.

ACKNOWLEDGEMENTS

We would like to thank A. Eady, C. Kamme and W. Wade for supplying a selection of *Propionibacterium* species. A. McD. and G. G. E. were funded by a Programme Grant from the Northern Ireland Health and Personal Social Services Research and Development Office (NIHPSS R & D Office). S. V. was funded by an NIHPSS R & D Office studentship and a grant from the British Orthopaedic Wishbone Trust. G. R. was funded by a Department of Employment and Learning Northern Ireland Studentship, M. M. T. was funded by the Arthritis Research Campaign, UK (project grant no. P0554), and S. O'H. was funded by Action Research, UK.

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