INTRODUCTION

*Bartonella henselae* is an ubiquitous flea-borne feline pathogen with a seroprevalence of 3–6% in blood donors in the USA and Australia (Branley et al., 1996; Chomel et al., 1995; Foley et al., 1998; Jackson et al., 1993; Koehler et al., 1994), which is capable of infecting other animals and causing serious illness in humans (Anderson & Neuman, 1997). Key virulence attributes probably include the ability to express a rigid polar (putative type IV) pilin (Batterman et al., 1997). Key virulence attributes probably include the ability to express a rigid polar (putative type IV) pilin (Batterman et al., 1997). Key virulence attributes probably include the ability to express a rigid polar (putative type IV) pilin (Batterman et al., 1997). Key virulence attributes probably include the ability to express a rigid polar (putative type IV) pilin (Batterman et al., 1997). Key virulence attributes probably include the ability to express a rigid polar (putative type IV) pilin (Batterman et al., 1997).

B. henselae is a fastidious, Gram-negative bacterial pathogen of cats and humans. Previous workers have shown that serial passage *in vitro* leads to attenuation of virulence-associated attributes such as expression of pili, invasion of human epithelial cell lines and the stimulation of endothelial cell proliferation. In contrast to the published data, it was found that pilin expression is frequently preserved in organisms which have undergone phase variation *in vitro*. Transition from a slow-growing, dry agar-pitting (DAP) to a faster-growing, smooth non-agar-pitting (SNP) form appears to occur predictably and may reflect competition between two populations growing at different rates. Better survival of the slower-growing (DAP) form may explain its relatively easy retrieval from piliated SNP populations allowed to age on solid media. Pilin expression is associated with auto-agglutination in liquid suspension or broth cultures, and appears to be necessary but not sufficient for expression of the agar-pitting phenotype and for the formation of biofilms. Outer-membrane protein variation is seen in association with phase variation, but lipopolysaccharide expression is preserved in piliated as well as extensively passaged non-piliated isolates. The Eagl/Hhal infrequent restriction site-PCR fingerprint, which has been previously used to discriminate between serotypes Marseille and Houston, is shown to alter with phase variation *in vitro*, and there is evidence that genetic change accompanies these events. The extent of genetic and phenotypic variability of phase-variant *B. henselae* has previously been underestimated. It may lead to new insights into the pathogenicity of this organism, and must be considered when interpreting data arising from such studies.

Phase variation generally refers to reversible change in a defined phenotype, often due to variable expression of important surface structures such as lipopolysaccharide (LPS), flagellae or adhesins (Henderson et al., 1999). Phase variations are usually random events, occurring at a relatively high frequency *in vitro* (\(>10^{-5}\) per generation), which may be influenced or modulated by environmental conditions (Gally et al., 1993). A slow-growing, agar-pitting, dry colony phenotype is reported to be typical of primary clinical isolates of *B. henselae* cultivated on chocolate agar plates (Arvand et al., 1998; Slater et al., 1990). Twitching motility is a characteristic feature of these isolates (Welch et al., 1992), as it is of other organisms expressing type IV pili (Tennant & Mattick, 1994). However, serial passage of dry agar-pitting isolates on solid media *in vitro* is associated with switching to a faster-growing, non-adherent and more-mucoid colony phenotype (Anderson & Neuman, 1997). This is associated with loss of pilin expression and the ability to invade human epithelial cells in tissue culture (Batterman et al., 1995), and to stimulate angiogenesis *in vitro* (Kempf et al., 2001). Most available data on the pathogenicity of *B. henselae* have been derived using the type strain (Houston-1), which is of variable and usually unstated passage number in different studies. Reversal of the reported attenuation with serial passage has not been formally described, and questions therefore remain about the interpretation of pathogenicity studies using stored or highly passaged isolates.

DNA fingerprinting methods have been used to distinguish...
subgroups within human \textit{B. henselae} isolates (Arvand et al., 2001; Dillon et al., 2002; Sander et al., 1998), and isolates with a 16S rDNA sequence matching that of the type strain (Houston-1) may be more common as a human pathogen (Dillon et al., 2002; Sander et al., 1999). Smal PFGE demonstrated at least seven different clonal types among 19 feline isolates of \textit{B. henselae} from Berlin, most of which were 16S type II (Arvand et al., 2001), and the question of inter-strain variability in virulence remains open (Dillon et al., 2002; O’Reilly et al., 1999; Relman, 1998; Chang et al., 2002). It may be that recombinatorial events, evident as phenotypic phase variation, comprise a basic virulence mechanism in \textit{B. henselae}, as described for other small vector-borne bacterial pathogens (Brayton et al., 2001). A bacteriophage has also been demonstrated in \textit{B. henselae} (Anderson et al., 1994). We have previously shown that all of 59 undifferentiated \textit{B. henselae} isolates were positive for PCR for the phage-associated \textit{papA} gene (Dillon et al., 2002), but this is of uncertain significance and it is unclear whether this gene is present in all primary isolates and whether it may be lost with serial passage \textit{in vitro}.

Two distinct serological types of \textit{B. henselae} have been reported (Drancourt et al., 1996), but inherent and phase-variable differences in LPS and outer-membrane proteins (OMPs) have not been systematically evaluated. There is considerable genetic heterogeneity within the species, but the extent to which genotypic and phenotypic variation confounds or informs the study of virulence remains unclear. A range of feline and human \textit{B. henselae} isolates from Australia and New Zealand were therefore evaluated in order to determine the extent to which phase variation influences \textit{in vitro} virulence-associated phenotypes of \textit{B. henselae}, including growth characteristics, biofilm formation, and the expression of major OMPs, pili and LPS.

This study was presented in part at the American Society for Rickettsiology/Bartonella Joint Conference 2001 (Big Sky, MT, USA; 17–22 August, 2001).

\textbf{METHODS}

\textbf{Bacterial strains and culture conditions.} A list of \textit{Bartonella} strains used in this study is given in Table 1. Unless stated otherwise, all strains were subcultured in the laboratory on chocolate blood agar (Oxoid Blood Agar base No.2) containing 5% (v/v) horse blood (CBA) at 35–37 °C in 5% CO\textsubscript{2} for 5–7 days, or grown in liquid broth medium (Schwartzman et al., 1993) at 37 °C in 5% CO\textsubscript{2} with shaking (100 r.p.m.) in cotton-wool-plugged narrow-necked pyrex Ehrenmeyer flasks (as specified in text). \textit{Salmonella} ser. Typhimurium (ATCC 14028) and \textit{Haemophilus influenzae} ATCC 10211 were grown on MacConkey agar and CBA, respectively, at 37 °C overnight. \textit{Vibrio cholerae} was grown at 37 °C using either T. C. B. S. Cholera Medium (Oxoid) or nutrient broth.

\textbf{Agglutination characteristics and sedimentation rates.} The experiment was performed in duplicate on three separate occasions. \textit{B. henselae} was inoculated from CBA into broth culture and incubated until an OD\textsubscript{600} value of between 0·65 and 0·85 was reached; optical density readings were taken using a Biophotometer 6131 (Eppendorf). \textit{Escherichia coli} DH5\textalpha was grown in nutrient broth and incubated under the same conditions. Clearing of cultures at 4 °C, 37 °C and room temperature was analysed for each strain as follows: aliquots of broth cultures were moved to stand for 4 h at the indicated temperatures. They were then vigorously vortexed for 10 s and allowed to settle. Duplicate 100 µl samples were gently removed from 10 mm below the surface of each of the broth samples after 20 min, and again 10 h later. Protein concentration was assayed using the Coomassie Plus protein assay (Pierce) in accordance with the manufacturer’s instructions.

\textbf{Growth curves of \textit{B. henselae}.} Growth and viability curves in isogenic sets were compared in broth media. Each strain was harvested from CBA and suspended in PBS to an OD\textsubscript{550} value of 0·25; duplicate broths were then inoculated 1:20 with the cell suspension. Aliquots were removed post-inoculation and then again every 24 h over the 10 day growth period. Bacteria were washed three times with PBS, then heated to 95 °C for 10 min and subjected to a freeze-thaw step using liquid nitrogen and a 65 °C water bath. Protein concentrations were determined using the Coomassie Plus protein assay.

\textbf{Enrichment of \textit{B. henselae} OMPs.} Preparation of membrane fractions was performed on 10 to 20 lawns of \textit{B. henselae} (per strain) which were washed three times with PBS (pH 7·4) before the cell

\textbf{Table 1.} \textit{B. henselae} strains used in this study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Source*</th>
<th>Type†</th>
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<tbody>
<tr>
<td>ATCC 49882 (Houston-1)</td>
<td>Human; ATCC</td>
<td>SNP</td>
</tr>
<tr>
<td>ATCC 49793</td>
<td>Human; ATCC</td>
<td>SNP</td>
</tr>
<tr>
<td>HC60 (S1–S5)‡</td>
<td>Feline; T. Gottlieb (Branley et al., 1996)</td>
<td>DAP</td>
</tr>
<tr>
<td>HC60′</td>
<td>HC60-derived (on 6th passage); this study</td>
<td>SNP</td>
</tr>
<tr>
<td>PK60 (S1–S7)</td>
<td>Recovered from aged HC60′; this study</td>
<td>DAP</td>
</tr>
<tr>
<td>PK60′</td>
<td>Derivative PK60; this study</td>
<td>SNP</td>
</tr>
<tr>
<td>NU4714 (S1–S3)</td>
<td>Feline; A. Morris (Joseph et al., 1997)</td>
<td>DAP</td>
</tr>
<tr>
<td>NU4714′</td>
<td>NU4714-derived (on 5th passage); this study</td>
<td>SNP</td>
</tr>
<tr>
<td>HC35 (S4–S6)</td>
<td>Feline; T. Gottlieb (Branley et al., 1996)</td>
<td>DAP</td>
</tr>
<tr>
<td>HC35 (S16–S20)</td>
<td>This study</td>
<td>DAP</td>
</tr>
</tbody>
</table>

*American Type Culture Collection, University Boulevard, Manassas, VA, USA; T. Gottlieb, Dept of Microbiology, Concord Hospital, Concord, Sydney NSW 2137, Australia. A. Morris, Dept of Microbiology, Green Lane Hospital, Auckland 1003, New Zealand.
†Colony morphology on chocolate blood agar.
‡Subculture (S) number (times subcultured or ‘passed’ \textit{in vitro}).
pellet was resuspended in 0.05 M HEPES buffer (pH 7.4) and lysed by sonication for 3–5 min on ice (Branson Sonic Power Sonifier 450; 60 % cycle, 20 % output). The sonicated suspension was freeze-thawed twice then centrifuged for 15 min at 3000 g (4°C). The supernatant was centrifuged for 45 min at 229 600 g (4°C) and the resulting pellet was resolubilized in 4·3 ml HEPES buffer. Forty-eight microlitres of 1 mM MgCl₂ and 0·48 ml of a 20 % (v/v) Triton X-100 solution were added to the mixture, which was then incubated at room temperature for 20 min. The sample was centrifuged for 45 min at 303 800 g (4°C). The insoluble pellet (outer-membrane fraction) was resuspended in HEPES (pH 7.4), and the protein concentration was determined using the Coomassie Plus protein assay. Bacterial membrane proteins were electrophoresed on SDS 4–20 % (w/v) polyacrylamide gels and stained with Coomassie brilliant blue, as described previously (Laemmli, 1970; Lugtenberg et al., 1975).

LPS preparation and LPS- (SDS) PAGE. Small-scale preparations of LPS were prepared by proteinase K digestion of whole-cell lysates, as described previously (Hitchcock & Brown, 1983; Morona et al., 1995) but with minor modifications. Five bacterial lawns per B. henselae strain were washed three times with PBS (pH 7·4) and pelleted by centrifugation for 2 min at 7000 g in 50 μl lysing buffer [2 % (w/v) SDS, 4 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol, 1 M Tris (pH 6·8) and 0·1 % (w/v) bromophenol blue]. The suspensions were boiled for 10 min, and protein was digested by the addition of 10 μl lysing buffer containing proteinase K (2·5 mg ml⁻¹; Astral Scientific); the suspensions were then incubated at 60°C for 5 h. Lysing buffer without proteinase K was added to paired controls before incubation. Bacterial LPS controls were also prepared as above (Salmonella ser. Typhimurium, full LPS; E. coli DH5α, rough LPS; H. influenzae, oligo LPS). Stored LPS preparations (4°C) were heated to 100°C for 5 min before SDS 20 % (w/v) PAGE with a constant current of 12–14 mA for 18–24 h; the tracking dye always yielded pure SNP populations (not shown).

Biofilm-formation assays. Experiments were performed in triplicate and repeated twice in a minor modification of an established method (Wättick et al., 1999). A clinical isolate of V. cholerae (El Tor) grown in plain nutrient broth (Voss & Attridge, 1993) and E. coli ATCC 25922 were utilized as controls (Pratt & Kolter, 1999). Ehrenmeyer flasks were utilized as surfaces for bacterial attachment. Duplicate broth cultures were incubated until an OD₅₅₀ value of 0·6–0·9 was reached. Each flask was rinsed vigorously to remove any non-adherent cells. A small loopful of selected biofilms was removed off the glass surface and analyzed by transmission electron microscopy. Each flask was filled with a 0·1 % (w/v) crystal violet solution, allowed to incubate for 30 min and then rinsed vigorously with distilled water. Biofilm formation was quantified by measuring the OD₅₇₀ value of a solution produced by extracting cell-associated dye with DMSO (Sigma).

Transmission electron microscopy (TEM). Preparation of nickel-coated 400 mesh grids for TEM was performed as described by Voss & Attridge (1993); the grids were counterstained with 1 % (v/v) uranyl acetate for 30 s before rinsing in distilled water.

PCR and sequencing: papA4, gltA4, 16S–23S rRNA intergenic region (ITS) and 16S rDNA. pap PCR was carried out according to previously published methods using Pap-1 and Pap-2 primers (Anderson et al., 1997). BH1 and BH2 primers were used in conjunction with the broad-host-range primer 16SF according to the method of Bergmans et al. (1996) for PCR and sequencing. 16S–23S ITS PCR was performed as described by Matar et al. (1993), with minor modifications. External primers RPC5 (5′-AAGGAGTGCCCGGAAGGTG-3′) and R23SS2693 (5′-TACTGTGTTCACTATGGTCTCA-3′) (200 nmol each) were used in a 100 μl PCR mixture (Matar et al., 1993). Internal primers BHITSF (5′-AAGGAGTGCCCGGAAGGTG-3′) and BHITSR (5′-GGTTTC-TTCGGCTTGTGCAA-3′) (Dillon et al., 2002) (200 nmol each) were used to amplify a partial ITS region for sequence analysis as follows: 5 min denaturation at 95°C; 35 cycles at 95°C for 30 s, 50°C for 60 s and 72°C for 90 s; final extension at 72°C for 10 min. Previously described primers (BHCs.1137, 5′-AATGGAAAAGGACAGTAAACA-3′; CS140f, 5′-TTACTATGATCCKGGYTATT-3′; 200 nmol each) were used to generate and sequence gltA amplicons (Birtles & Raoult, 1996).

Infrequent restriction site (IRS)-PCR with Eagl/HhaI. This method employs the use of an infrequent (Eagl) and a frequent (HhaI) restriction enzyme to digest genomic DNA, which is then amplified with primers and adapters that target the extremities of the restricted fragments (Riffard et al., 1998). It offers a robust PCR-based tool for sampling restriction sites of choice across the genome. By amplifying only those fragments that have both restriction sites, it generates less bands and thus easier analyses than a PFGE-based approach using the same enzymes. PCR reagent concentrations and amplification conditions have been described by Handley & Regnery (2000). DNA amplification was performed in a PC-960C thermal cycler (Corbett Research). PCR products were separated on a 6·5 % (w/v) polyacrylamide gel in 1× TBE buffer for 3 h at 150 V, and visualized using ethidium bromide staining and an ultraviolet transilluminator using 667 Polaroid film.

Statistical analysis. Data were analysed by the Student’s t test using the SPSS package (version 11.0.1) for Windows.

RESULTS

Predictable changes in growth characteristics occur with serial passage on solid media

Colony morphology on chocolate blood agar was observed to be generally one of two types, as previously described (Batterman et al., 1995). A smooth non-agar-pitting (SNP), domed colony type was most common in our isolates, including those of apparently low passage number (<54) (Table 1). A dry agar-pitting (DAP) colony morphology was observed in several isolates (HC35, HC60 and NU4714) and was lost in almost all isolates on repeated passage (>54), also as previously described (Batterman et al., 1995), and these were selected for study. We confirmed previous reports that the DAP type gave rise on repeated passage after a few days growth in vitro to the SNP colony type, and found that this occurred at a predictable and reproducible passage number. At the given (transition) passage number (Table 1), subcultures yielded a mixed population of vigorously growing SNP colonies which were easily swept from the agar with a loop, and smaller, drier DAP colonies which appeared to corrode the agar surface and were difficult to remove with a loop, as well as occasional colonies of hybrid type. The proportions of the two colony morphologies were typically approximately equal at the transition stage, with SNP colonies invariably becoming apparent first. A DAP colony picked from such (transitional) agar plates yielded the same equal proportions of SNP and DAP colonies on subculture, while SNP colonies always yielded pure SNP colonies which always yielded pure SNP populations (not shown).
DAP revertants can be derived from phase-variant colonies

Occasional sectored colonies and frequent dry, granular outgrowths from aged SNP colonies were observed in many subcultures, especially those that had recently undergone transition. HC60’ (the prime symbol denoting the transition to SNP), NU4714’, ATCC 49882 (Houston-1) and ATCC 49793 were therefore aged on chocolate blood agar for 18–45 days. Sectoring and outgrowths were not observed in our culture of Houston-1 (SNP type) nor in our culture of ATCC 49793 (SNP type), with these two strains being of relatively high passage number. While aged (> 30-day-old) colonies of the SNP phenotype HC60’ (S12) reliably yielded a DAP form (which we named PK60) on subculture, DAP forms could not be similarly obtained from aged SNP colonies of NU4714’ on several attempts. PK60 behaved essentially in the same way as HC60 (its DAP phenotype switching after six further subcultures), and the SNP phenotype of PK60’ remained stable with further rapid passage in vitro. An agar-pitting (DAP) form of B. henselae Houston-1 was recovered after passage of 29-day-old colonies onto fresh chocolate blood agar. The small dry colonies formed deep pits in the solid agar, but subculture of a sweep of these colonies yielded only SNP forms again on solid agar. A stable DAP variant was not obtained on three attempts. We were unable to select agar-pitting isolates from aged SNP forms of NU4714 (SNP type), with these two strains being of relatively high passage number. While aged (> 30-day-old) colonies of the SNP phenotype HC60’ (S12) reliably yielded a DAP form (which we named PK60) on subculture, DAP forms could not be similarly obtained from aged SNP colonies of NU4714’ (Fig. 1). SNP forms consistently grew faster than isogenic DAP parent cultures, with similar results obtained for all SNP and DAP isolates. Initial subcultures of HC35 (e.g. S4) grew slower than those of higher passage number (e.g. S20), despite persistence of the DAP phenotype, suggesting that the changes in growth rate may be independent of the change in colony phenotype. Furthermore, the most striking difference was seen in the NU4714/NU4714’ pairing, in which reversibility of the phenotype could not be demonstrated.

Temperature-dependent auto-agglutination is not directly related to phase variation

Variable, coarse auto-agglutination in liquid culture was observed at different temperatures and between isolates. All cultures cleared on standing for 10 h, the rate of sedimentation at 37˚C being generally much faster among DAP cultures [HC35 (S4), HC35 (S20), NU4714 (S2), HC60 (S2) and H-1] than SNP cultures [NU4714’ (S12), HC60’ (S12) and ATCC 49793]. While sedimentation of HC35 was little changed from S4 to S20 (93 % vs 88 % precipitating from suspension in 10 min), a significant decrease in the rate of precipitation of biomass out of suspension was observed in paired sets. Mean percentage precipitated in 10 min changed from HC60 (71 %) to HC60’ (23 %) and back to PK60 (89 %), as well as for NU4714 (86 %) to NU4714’ (10 %), with $P < 0.05$ for HC60/HC60’, HC60’/PK60 and NU4714/NU4714’ comparisons. However, there was an imperfect correlation between agglutination and agar-pitting phenotypes. PK60 sedimented faster than the original DAP parent HC60 (S2), and while our copy of the Houston-1 ‘type’ strain did not pit solid agar on routine culture, it coarsely agglutinated in liquid culture (75 % in 10 min) as well as when resuspended in PBS after scraping from plates (not shown). Holding exponential-phase cultures at different temperatures for 4 h also affected the sedimentation rate, with most agar-pitting strains sedimenting more rapidly after they had been standing at 37˚C than after standing at 4˚C. Interestingly, the converse was true for non-agar-pitting strains, with one exception, namely Houston-1. Intermediate results between agar-pitting and non-agar-pitting strains were found at 25˚C (data not shown).

Biofilm formation by B. henselae in vitro

Biofilm formation has not been previously characterized in B. henselae, although early observers noted adherence of the organism to flasks (Regnery et al., 1992). Formation of a wash-resistant biofilm on the inert (pyrex) surface of Ehrlenmeyer flasks was greatest in strain HC35, while NU4714’ (S12) formed the smallest biofilm out of the strains tested (Fig. 2).

Piliation may be preserved with phase variation

Pili were readily detected in all DAP forms and some, but not all, SNP forms of B. henselae (Fig. 3). Piliation was preserved in strain Houston-1 and in the SNP form of HC60 (HC60’), but it was much reduced in NU4714’ (S12) (Fig. 3) and
ATCC 49793 (not shown), both of which agglutinated less heavily and formed poor biofilms. Bundled pili were also apparent on organisms collected from adherent Houston-1 biofilms (derived from subculture of SNP forms into broth) and in the surrounding biofilm medium (data not shown).

Phase variation is not due to loss of LPS expression

Analysis of LPS by SDS-PAGE and silver staining disclosed no difference between phase-variant pairs NU4714 (S2)/NU4714′ (S12) and HC60 (S2)/HC60′ (S12) nor the early-(S4) and late-passage (S20, persistently DAP) forms of HC35 (Fig. 4). Minor variation in the LPS chain length of the two ATCC strains (ATCC 49793 and ATCC 49882) was suggested, but there was no loss of LPS expression. Salmonella ser. Typhimurium, H. influenzae and E. coli DH5α (deep, rough) were used as comparators.

Phase variation is associated with minor alterations in OMP expression

There was a subtle variation in the expression of a number of proteins in detergent-insoluble fractions of whole-cell lysates, but variation in OMP fractions was most prominent for a protein of approximately 23 kDa (Fig. 5) in phase-variant forms of strains NU4714 and HC60 (light arrow, Fig. 5). This small protein was also visible in Houston-1 (lane 9, Fig. 6). A prominent 43 kDa band facilitated comparison (dark arrow, Fig. 5).

Phase variation may be accompanied by genetic alteration

The papA gene was detected by PCR in all isolates tested, including NU4714′ (S12), HC60′ (S12), PK60 (derived from aged SNP forms of HC60′ and PK60′ (S9) (not shown). No plasmids were detected on agarose gel electrophoresis of whole-DNA extracts prepared from agar-pitting cultures of HC60, NU4714 or HC35 (not shown). The 16S type, 16S–235 intergenic sequence (ITS) region and the gltA gene were all unaffected, as were the (M13 core primer-based) AP-PCR and ERIC-PCR ‘fingerprint’ pattern types (data not shown). However, analyses of the genomes of the strains by Eagl/HhaI IRS-PCR suggested genomic rearrangements that might be relevant. A small band at ca. 350 bp was lost from HC60 when it changed to the SNP form (HC60′), which was then regained in PK60 and lost again in the phase-variant strain PK60′ (Fig. 6). A different change was seen in NU4714 when it changed to the SNP form (NU4714′), while HC35 altered little or not at all in its phenotype and no genetic alteration was detected in the Eagl/HhaI IRS-PCR analysis. It is important to note that our copy of Houston-1 (SNP) had the same IRS-PCR pattern as NU4714′ (S12) but not ATCC 49793 (both of SNP phenotype), and that we could not obtain a stable DAP form from any of these strains.

**DISCUSSION**

ATCC 49793 and its isogenic parent strain B. henselae 87-66 (Welch et al., 1992) have been previously compared as an illustration of phase variation in B. henselae, defined in terms of in vitro growth characteristics, and associated with loss of piliation and invasiveness (Batterman et al., 1995). Extensive passage in vitro has also been shown to attenuate the angiogenic phenotype (Kempf et al., 2001). We have confirmed that phase-variant (SNP) forms are indeed faster growing and may out-compete the DAP parent in nutritious artificial media, but DAP forms appear to have the advantage in aged cultures. We have also shown here that the phase-variation phenomenon is less straightforward than previously suggested and is not necessarily associated with loss of pilin expression or with loss of pilin-associated phenotypes such as biofilm formation.

We compared three sets of organisms, including one that was reversibly phase-variant, and another in which the ‘original’ DAP phenotype persisted upon multiple passages. We also found that the two ATCC strains we obtained were unable to stably maintain a DAP phenotype. One of these (ATCC 49793) is the ‘quality assurance’ strain widely used in Australian diagnostic laboratories and we know this strain to have been extensively passed in vitro. Outer-membrane profiles were relatively similar between all strains tested. However, SNP isolates HC60′, NU4714′ and Houston-1 all have a prominent band at approximately 23 kDa. This band does not appear in HC35 (S20), nor is it present in the heavily passaged strain ATCC 49793. Strain ATCC 49793 is clearly quite attenuated, with loss of pilin expression and associated phenotypes, and therefore may not reliably reflect early changes associated with phase variation (as seen in HC60′ and NU4714′). Whether the 23 kDa OMP seen on SDS-PAGE analysis reflects a compensatory or regulatory event remains to be determined, but there is evidence of genetic change or rearrangement occurring in the strains presenting this protein. IRS-PCR with Eagl/HhaI has been used to separate the DNA ‘fingerprints’ of Houston-1 and Marseille (Handley & Regnery, 2000). We find, however, that the distinguishing (ca. 200 bp) band for Houston-1 is in fact absent from our copy of Houston-1. It is present in...
ATCC 49793, which has Houston-type 16S rDNA and gltA sequences, but is also absent from NU4714'. Like Houston-1, NU4714' never yielded stable DAP revertants. Another, more subtle, band (ca. 350 bp) appears to come and go in the HC60-derived isolates, suggesting a relevant genetic change, but one which does not affect the presence or amplicon size obtained from the papA gene. The exact significance of such changes and the extent to which they are

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**Fig. 3.** Piliation of *B. henselae*. (a) HC60, (b) HC60', (c) PK60, (d) PK60', (e) NU4714 and (f) NU4714'. Bars, 0.5 μm.
useful guides to phase variation is uncertain, but emphasizes that morphological change is associated with one or more genetic events. Comparison with NU4714 demonstrates that these events are not identical and that they may not be causal.

Our copy of Houston-1 is clearly a non-agar-pitting form of \textit{B. henselae}, but it is well piliated, strongly auto-agglutinates and forms a biofilm. This contrasts with the poorly piliated strain ATCC 49793, which does not auto-agglutinate or form a biofilm, consistent with a pilin-dependent agar-pitting or twitching phenotype but illustrating that adhesins sufficient to promote strong intercellular and inert surface attachment may be present in its absence. Biofilm formation is likely to be a basic characteristic of \textit{B. henselae}, as suggested in initial descriptions of the organism (Regnery \textit{et al.}, 1992). Pilin expression appears to be necessary for biofilm formation, and the associated \textit{in vitro} phenotype may alter with serial passage in a way that is influenced by auto-agglutination, with coarse granules precipitating out of solution. The agar-pitting phenotype is most likely to be a reflection of solid-phase (twitching) motility, and pili are necessary but insufficient for this phenotype. The ability to migrate to fresh nutrient sources may thus explain the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{LPS expression in \textit{B. henselae}. LPS- (SDS) PAGE. Lanes: 1, \textit{Salmonella} ser. Typhimurium ATCC 14028; 2, \textit{E. coli} DH5\textsubscript{x}; 3, \textit{H. influenzae} ATCC 10211; 4, NU4714; 5, NU4714'; 6, HC60; 7, HC60'; 8, empty; 9, HC35 (S4); 10, HC35 (S20); 11, ATCC 49882 (Houston-1); 12, ATCC 49793; 13, lysing buffer minus proteinase K (control).}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Coomassie-blue-stained SDS-PAGE \[4–20\% (w/v) gradient acrylamide gel\] analysis of the Triton X-100-insoluble outer-membrane fraction. Lanes: 1, high-molecular-mass standards; 2, low-molecular-mass standards; 3, NU4714; 4, NU4714'; 5, HC60; 6, HC60'; 7, HC35 (S4); 8, HC35 (S20); 9, ATCC 49882 (Houston-1); 10, ATCC 49793. Protein (90 \mu g) was loaded into each well. Mass values of the protein standards are numbered on the left for convenient reference. The 43 kDa Omp43 is marked with a dark arrow and the 23 kDa region is marked with a light arrow.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Eagl/HhaI IRS-PCR. (a) Lanes: 1, ATCC 49882 (Houston-1); 2, NU4714; 3, NU4714'; 4, ATCC 49793; 5, HC35 (S4); 6, HC60'; 7, HC60. (b) Lanes: 8, PK60; 9, PK60'. (c) Lanes: 10, HC35 (S4); 11, HC35 (S20). M, pGEM DNA size marker (kbp; Promega).}
\end{figure}
observed selection of DAP forms in those (piliated) SNP forms that retain the potential for reversion, and this may occur as a reversible genetic event, accompanied by direct or compensatory alterations in the expression of specific OMPs. The predictable development of SNP forms from DAP forms may be explained by competitive success of the faster-growing (SNP) forms in nutritious media, but (presumably without any solid-phase motility) exhausting nutrient supply and dying in aged colonies while slower-growing pitting/spreading DAP forms persist. Ultimately, an event resulting in loss of pilin expression or other irreversible events may make the return to the DAP form impossible. This is consistent with observations that primary isolates which may be detected in tissue culture are poorly recovered on solid media (La Scola & Raoult, 1999) and with pili of a type which, in other organisms, confer solid-phase (twitching) motility (Batterman et al., 1995). Thus, the passaged, non-reverting strains may include phase-variant isolates with acquired secondary and/or compensatory mutations, some of which may make it impossible to revert to the DAP phenotype.

In conclusion, and in contrast to published data, it is now clear that the growth characteristics associated with phase variation in B. henselae are not necessarily associated with loss of piliation, may be associated with variation in OMP expression and apparent genotype, and are variably reversible. Biofilm formation and expression of putative type IV pili appear to be basic characteristics of this organism. Genetic markers previously thought to distinguish between serotypes Houston and Marseille are shown to be misleading and probably reflect unrelated genetic events which may yet give us some insights into phase variation in B. henselae. Future work should be directed at understanding the genetic basis of phase variation in B. henselae, and detailed studies of proven primary isolates are needed. Workers in the field are cautioned to consider the genetic and phenotypic stability of isolates from which data regarding virulence and pathogenicity are generated.

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REFERENCES


Phase variation in Bartonella henselae


