Mechanisms Underlying *Campylobacter fetus* Pathogenesis in Humans: Surface-Layer Protein Variation in Relapsing Infections

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*Campylobacter fetus* causes gastrointestinal and systemic infections in humans. Although relapse is common despite antibiotic treatment, the mechanisms are not well understood. The surface-layer proteins (SLPs) of *C. fetus*, which are critical in virulence, undergo high-frequency phenotypic switching due to recombination of *sap* homologues, resulting in antigenic variation. To investigate the mechanisms involved in relapsing *C. fetus* infections, we compared SLP variation in 4 pairs of *C. fetus* strains that infect humans; initial and follow-up isolations were performed 20 days to 34 months apart. Of the 4 pairs of strains, 2 had antigenic variation, and another provided evidence for selection for SLP-positive populations. Southern hybridization indicated recombination underlying the SLP variation and up-regulation. The fourth pair had the same SLP antigenic profile and *sap* homologue hybridization pattern, which is consistent with latency of the original strain in a privileged locus. In total, these findings indicate that relapse may reflect at least 3 differing pathogenetic pathways.

The ability of pathogenic microbes to persist in vertebrate hosts that are protected by adaptive immunity is a central biological problem. Generation of antigenic variation of surface components is one of the mechanisms enabling evasion of host immune responses during long-term colonization [1]. Rearrangement of the genetic loci involved in expression of major antigens, an efficient mechanism for generation of diversity, is employed by a wide range of microbes [2–6].

*Campylobacter fetus*, a gram-negative bacterium, is capable of persistently colonizing vertebrate hosts and also can cause infections both in animals important for food production and in humans [7–14]. Surface-layer proteins (SLPs), which are encoded by the *sap* homologues, are critical virulence factors during *C. fetus* infection [15–21]. In wild-type *C. fetus* strain 23D, the 9 (8 complete and 1 partial) *sapA* homologues are clustered on a 54-kb chromosomal DNA locus (termed the “*sap* island” [22, 23]) that surrounds the genes required for secretion of the SLPs from the bacterial cell [24].

Genetic recombination resulting in phenotypic variation can occur at high frequencies within the *C. fetus* *sap* island [25–29]. Recombination among the multiple *sapA* homologues, mediated by their 5′ conserved regions [26, 28], results in phase variation in expression of homologues; the homologue located downstream of the unique *sap* promoter can be transcribed, whereas all other *sapA* homologues are transcriptionally silent [30–32].

*C. fetus* can persistently colonize its natural mammalian hosts [29, 33, 34]. The high-frequency DNA rearrangement within the *sap* island, leading to antigenic variation, implies that persistence may be facilitated by the organism’s ability to adapt to dynamic environments by continuous change. In the present study, we investigate whether antigenic variation can be observed in 4 pairs of *C. fetus* strains isolated from human...
Mechanisms of *C. fetus* Variation

### Table 1. Characteristics of the *Campylobacter fetus* strains examined in the present study.

<table>
<thead>
<tr>
<th>Pair (isolation interval), strain</th>
<th>Designation</th>
<th>Source</th>
<th>SLP type</th>
<th>Size of SLP,$a$ kDa</th>
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<tr>
<td>1 (5 months)</td>
<td>CA700</td>
<td>Synovial fluid</td>
<td>A</td>
<td>97, 127</td>
</tr>
<tr>
<td>2 (20 days)</td>
<td>CA725</td>
<td>Synovial fluid</td>
<td>A</td>
<td>97, 127</td>
</tr>
<tr>
<td>3 (1–2 months)</td>
<td>29826</td>
<td>Blood</td>
<td>A</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>CA740</td>
<td>Blood</td>
<td>A</td>
<td>97</td>
</tr>
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<td>5 (3 months)</td>
<td>51560</td>
<td>Blood</td>
<td>A</td>
<td>127</td>
</tr>
<tr>
<td>6</td>
<td>51918</td>
<td>Blood</td>
<td>A</td>
<td>149</td>
</tr>
<tr>
<td>NA (control)</td>
<td>23D</td>
<td>Vagina (cow)</td>
<td>A</td>
<td>97</td>
</tr>
<tr>
<td>9 (24 months)</td>
<td>84107</td>
<td>Blood</td>
<td>B</td>
<td>97</td>
</tr>
</tbody>
</table>

**NOTE.** NA, not applicable; SLP, surface-layer protein.

### Table 2. Polymerase chain reaction primers used in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Orientation</th>
<th>5′ position$^a$</th>
<th>Sequence (5′→3′)</th>
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<tr>
<td>A0F</td>
<td>sapA</td>
<td>F</td>
<td>1903</td>
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<tr>
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<td>F</td>
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<td>A1R</td>
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<td>sapA2</td>
<td>F</td>
<td>2741</td>
<td>CTAAACTAAAACGATTTG</td>
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<td>A2R</td>
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<tr>
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<td>2496</td>
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<tr>
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<td>2783</td>
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<tr>
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<td>sapA6</td>
<td>R</td>
<td>3562</td>
<td>TTGACTCCTTTTTGCTT</td>
</tr>
<tr>
<td>7F1</td>
<td>sapA7</td>
<td>F</td>
<td>3262</td>
<td>ATGTGAAGGTACAAAGA</td>
</tr>
<tr>
<td>7R1</td>
<td>sapA7</td>
<td>R</td>
<td>3850</td>
<td>CGTCTAAAGCTGATTAA</td>
</tr>
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<td>1</td>
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<td>sapA</td>
<td>R</td>
<td>531</td>
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<tr>
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<td>sapB</td>
<td>F</td>
<td>20</td>
<td>TTCAAGAGTTTTTAGTTC</td>
</tr>
<tr>
<td>SBR01</td>
<td>sapB</td>
<td>R</td>
<td>524</td>
<td>TCAACACATATTACTAAC</td>
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</tbody>
</table>

**NOTE.** All primers are from [38], except SAF01, SAR01, SBF01, and SBR01, which are from [37]. F, forward; R, reverse.

$^a$ Position in open-reading frame, according to sequence for strain 23D (GenBank accession no. AE211269).

Patients with relapsing infections. Despite seemingly effective antibiotic therapies, relapsing *C. fetus* infections in humans have been well observed [35]. We now report analyses of genotypic and phenotypic variation of paired *C. fetus* strains from 4 patients with relapsing infections. The present study provides a better understanding of the mechanisms underlying recurrent infections.

### MATERIALS AND METHODS

**Strains.** All 4 pairs of *C. fetus* strains were isolated from patients in the province of Quebec, Canada. For each pair of strains, the clinical characteristics and the interval between isolations was known (table 1). In the present study, *C. fetus* strains were grown at 37°C under microaerobic conditions, in GasPak jars, by use of a CampyPak Plus gas generator (BBL Microbiology Systems), on brucella agar (Difco Laboratories) containing antibiotics at the following concentrations: 7 U/mL polymyxin B, 10 μg/mL vancomycin, 15 μg/mL nalidixic acid, and 10 μg/mL trimethoprim lactate.

**Polymerase chain reaction (PCR) techniques.** The PCR primers used in the present study are listed in table 2. To characterize the strains as either type A or type B, *sapA*-specific (SAF01 and SAR01) and *sapB*-specific (SBF01 and SBR01) primers, respectively, were used [36, 37] (table 2). To ascertain the presence of particular *sap* homologues, homologue-specific primers (A0R–7R1) (table 2) were used for PCR amplifications [38]. PCR was performed in reaction volumes of 50 μL containing 0.25 U of *Taq* polymerase (Qiagen), 10 mmol/L MgCl₂, and 10 pm of each primer. The PCR protocol (35 cycles) in-
Figure 1. Identification of the sap type and distribution of the 8 studied *Campylobacter fetus* strains. Polymerase chain reaction (PCR) was performed by use of 5′ conserved-region primers from *sapA* (A) or *sapB* (B). DNA from strains 23D and 84-107 were used as type A and type B sap homologue controls, respectively. Identification of the sap homologues present, detected by sap homologue–specific PCR (C), was based on the defined *sapA* homologues in strain 23D. DNA from strains 23D and 23B was used as controls.

Southern hybridization. Chromosomal DNA was prepared from *C. fetus* cells grown on 2 plates for 48 h, by use of the Wizard Genomic DNA Purification Kit (Promega). DNA was digested with *Hind*III, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane (MSI). The hybridization probe was a PCR product amplified by use of the *sapA* 5′ conserved-region primers [37] or promoter-specific primers [30], with template DNA from strain 23D. Probes were labeled by use of the Renaissance chemiluminescent nonradioactive kit (NEN Research Products).

RESULTS

C. fetus strain typing. *C. fetus* SLPs may be type A, type B, or, occasionally, type AB, which is consistent with determination of their serotype and lipopolysaccharide type [36, 37]. To characterize the 4 pairs of strains, we performed PCR using type A (*sapA*-specific) or type B (*sapB*-specific) primers. The results showed that all 4 pairs of *C. fetus* strains from these systemic infections of humans were type A, and not type B or type AB (figure 1A and 1B).

Repertoire of sap homologues for each pair of strains.
Variation of C. fetus SLP expression, mediated by DNA recombination, is due to rearrangement of the unique sap promoter upstream of one of the multiple sap homologues [30]. To determine the repertoire of sapA homologues contained within the sap locus, for each of the 4 pairs of strains, we performed PCR using primers specific for each of the 8 sap homologues that exist in strain 23D [22, 38]. The PCR results showed that the same sapA homologues were present in each pair of strains (figure 1C). Three pairs possessed all 8 sapA homologues that exist in strain 23D [38], but the pair-2 strains did not possess sapA. Thus, the 4 sets of strains were relatively conserved in the sap homologues that they possessed.

To characterize the arrangement of the sap locus in each pair of stains, we performed Southern hybridization using as a probe the 5′ conserved region present in all sapA homologues. These results (figure 2A) indicated that each pair shared a characteristic overall hybridization pattern, but that, in pairs 1, 2, and 3, rearrangements had occurred between the strains. In pair 1, strain 2 (CA725) gained 2.7-kb and 2.9-kb bands, compared with strain 1 (CA700). In pair 2, strain 4 (CA740) gained a 4.2-kb band, compared with original strain 3 (29826). In pair 3, strain 6 (51918) lost a 4.2-kb band, compared with strain 5 (51560). For the pair-4 strains (designated “51534” and “CA1124”), there were no differences in sap hybridization patterns. Southern hybridization using the sap promoter as a probe showed that the promoter had been rearranged within populations of the second strains of pairs 1, 2, and 3, compared with populations of their first strains (figure 2B). From these studies, we concluded that, in pairs 1, 2, and 3, the strains were clonal variants of one another, whereas there was no evidence for variation in the populations of the pair-4 strains.

The expressed SLPs. To examine the phenotype of each pair of strains in terms of SLP expression, we performed SDS-PAGE analysis and then performed immunoblotting using polyclonal antiserum raised to the 97-kDa type A SLP [39] (figure 3). Each pair of strains had the same protein profile, as determined by SDS-PAGE analysis, with the exception of the SLP bands (figure 3A). Immunoblotting indicated that, in pair 1, strain 2 had 2 major bands (97 and 127 kDa), whereas strain 1 had a major band (127 kDa) and a minor band (97 kDa) (figure 3B). In pair 2, strain 3 had a minor band (97 kDa), whereas strain 4 had a major band (97 kDa). In pair 3, both strain 5 (127 kDa) and strain 6 (149 kDa) had a major band. In pair 4, strains 7 and 8 had a major band of the same size (97 kDa).

Since both strains in pair 1 had 2 SLP bands, these could be accounted for by either a mixture of 2 populations of cells expressing different SLP bands or a single population simultaneously expressing 2 different major bands. To address these possibilities, we examined individual colonies of both strains. All 46 colonies of strain 1 (CA700) had the same 97-kDa band;
however, 35 colonies had an extra 127-kDa band, and the other 11 colonies had a 131-kDa band (figure 4A). Of 18 colonies of strain 2 (CA725), 13 had a major 97-kDa band, 2 had major 127-kDa bands, and the other 3 did not have any SLP bands (figure 4B). Thus, there was extensive phenotypic (SLP) diversity within the population of cells in pair 1. In pair 2, strain 3 (29826) had a minor 97-kDa band, whereas strain 4 (CA740) had a major 97-kDa band. On the basis of these findings and the findings of Southern hybridization using the promoter probe (figure 2B), we hypothesized that, in the pair-2 populations, the SLP promoter may be subject to high-frequency rearrangement events. Examination for SLP expression showed that none were detected in the 13 colonies of strain 3 (29826) cells or in 4 of the 13 colonies of strain 4 (CA740) cells (figure 4C). These findings suggest that deletions involving the sapA promoter may have been occurring at a high frequency in strains from this pair.

**Serum resistance of the pair-2 strains.** All pathogenic *C. fetus* strains are SLP positive [15]. If the capacity to express SLPs is removed, then the resulting strains are markedly reduced in virulence in vitro [15] and in vivo [16, 18]; SLP-
Mechanisms of C. fetus Variation

Figure 5. Comparison of serum susceptibility and the profiles of the sapA homologues for pair-2 strains and their derivatives. A, Serum susceptibility of the selected Campylobacter fetus strains. Strains were incubated for 60 min with 10% normal human serum, as described in Materials and Methods, and survivors were enumerated. Strains are original strain 3 (29826), strain-3 variants 3S+ (SLP positive) and 3S- (SLP negative), original strain 4 (CA740), strain-4 variant 4S+ (SLP positive), and control strains 23D and 23B. B, Southern hybridization of HindIII-digested genomic DNA probed with sapA 5′ conserved region. C, Polymerase chain reaction amplification with sapA4-specific primers (A4F and A4R). Lane C represents the no. of the DNA PCR control.

negative strains are sensitive to NHS, whereas SLP-positive strains are resistant to NHS [15]. To further assess the population differences in SLP expression between pair-2 strains 3 (29826) and 4 (CA740), the cells were incubated with NHS to quantitate resistance to NHS. As expected [40], strain 23D was resistant to NHS (>$10^{-1}$ survival), whereas SLP-negative strain 23B was highly sensitive to NHS (<$10^{-3}$ survival). After incubation with NHS, the survival rate for strain 3 was $10^{-2.6}$, whereas, for strain 4, it was $10^{-0.8}$, nearly $2 \log_{10}$ higher (figure 5). For each of the strains, we selected 10 survivors of incubation with NHS to examine SLP expression. All 10 colonies of NHS-resistant strain 4 expressed 97-kDa SLPs, whereas only 2 of 10 colonies of strain-3 NHS survivors expressed 97-kDa SLPs; the other 8 were SLP negative (data not shown). To determine whether the shift to SLP negativity could be completely explained as its being an in vitro phenomenon, we selected 1 colony each of SLP-positive strain-3 and strain-4 survivors. Both continued to be highly resistant to NHS ($>10^{-1}$ survival), similar to strain 23D (figure 5A). We next examined the susceptibility to NHS of serum-surviving but SLP-negative strain 3. After incubation with NHS, the survival rate was $10^{-2.4}$, nearly identical to the result for its parental strain and much higher than that observed for the spontaneously mutated SLP-negative strain 23B ($<10^{-3}$ survived) (figure 5A). These results show that strain 3 included several populations that differed in SLP expression and in resistance to NHS. A much greater proportion of the cells from subsequent strain 4 were high-level SLP expressers and were ~100-fold more resistant to NHS. The results of analysis of this pair of strains suggested that there had been in vivo selection for resistance to NHS.

Genetic characteristics of the pair-2 variants. To investigate the basis for why pair-2 strains and their derivatives had different susceptibilities to NHS, we compared the sap homologue profiles among single colonies of strains 3, 3S- (re-
sistant to NHS and SLP negative), 3S+ (resistant to NHS and SLP positive), 4, and 4S+ (SLP positive). The results indicated that strain 3S+ lost a 4.2-kb band, compared with strain 3S−. As expected, the 4.2-kb band also existed in strain 4 and its selected clone, 4S+, and was not detected in original strain 3 (figure 5B). The sap homologue-specific PCR indicated that sapA4, the SLP-expressing gene in strains 3S+ and 4S+, was deleted in strain 3S− (figure 5C).

To determine whether strain 3S− underwent genotypic and phenotypic variation over time, we passed strain 3S− 10 times in vitro, representing hundreds of generations. We performed Western blotting and PCR for strains 3S− and 3S+, at 0, 5, and 10 passages. By Western blotting, each strain had no change in original SLP phenotype (data not shown). By PCR, strain 3S− had the expected products in the promoter PCR and in the sapA4 PCR, without change from passage 0, 5, or 10. For strain 3S+, there were no PCR products at passage 0, 5, or 10, also as expected (data not shown). These data confirm the in vitro stability of genotypes and phenotypes related to SLP expression and provide further evidence that the changes we observed with the passed strains reflected in vivo phenomena.

**DISCUSSION**

We have characterized 4 pairs of *C. fetus* strains from humans with relapsing infection by comparing their sap type, sap island repertoire and organization, and the SLPs expressed. In each case, both strains in the pair were type A, which appears to be more common in human infection [37], although no systematic study has been done. That 3 pairs of strains possessed all 8 sapA homologues that exist in type A strain 23D and that both strains in the other pair possessed 7 sapA homologues (without sapA) suggest conservation of this particular repertoire of multiple sap homologues among the *C. fetus* strains infecting humans, a finding that is consistent with our previous observations of 18 *C. fetus* strains from a variety of sources [37]. Whether the strains we studied possessed any of the 5 other sapA homologues that have been characterized from other *C. fetus* isolates [38] was not determined.

The sapA 5′ conserved-region Southern hybridization (figure 2A) reflected the organization of the sapA homologues, in relation to their genomic positions. All 4 pairs of strains had different sap island profiles, indicating that each had a different epidemiologic source, but, within each pair, there was a highly similar pattern of organization of the sap island, confirming that both strains in each pair had the same ancestor.

Our results indicate that, in 3 of the 4 pairs of strains studied, the strain isolated after relapse of *C. fetus* infection had undergone change in the predominant SLP expressed. This was illustrated in the pair-1 strains, for which the overall (figure 3) and the detailed (figure 4) analyses showed differences in size and amount of SLP expression. Southern hybridization indicated one basis for these changes: differing rearrangements involving the sapA homologues and the sap promoter. In pair 3, strain 6 produced a 149-kDa SLP instead of the 127-kDa SLP produced by strain 5, a finding consistent with selection for antigenic variation to escape host immune defenses. That the pair-4 strains demonstrated the same SLP expression illustrates a different phenomenon. Strain 7 was isolated from the blood of a patient with septicemia who had been successfully treated with antibiotics. Weeks later, the patient developed chronic arthritis from which synovial fluid was cultured several times; this fluid was found to be positive for *C. fetus* only once, 34 months later. This long interval is most consistent with latency of low-grade *C. fetus* infection in a privileged site and then reactivation of the original strain with a change in host status, as has been observed previously [34]. That the pair-4 strains appeared to be identical at both phenotypic and genotypic levels supports the hypothesis of latency, paralleling that documented for other infectious agents, including *Mycobacterium tuberculosis*, *Herpes* viruses, *Plasmodium* species, and *Rickettsia prowazekii*.

Our analyses of the pair-2 strains provide a new understanding of *C. fetus* virulence. The first isolate, strain 3, showed little SLP expression, and Southern hybridization did not detect the sap promoter. For this strain, the susceptibility to NHS was intermediate between that of strain 23D and that of the spontaneous SLP-negative strain 23B, in which the sap promoter had been deleted [32]. This suggests the possibility that the strain actually represented a mixture of 2 such populations, with high-frequency switching. The subsequent SDS-PAGE and serum-susceptibility analyses confirmed the existence of 2 such populations that differed in whether they expressed SLP. Within that mixture, a completely resistant SLP-positive population was present. We hypothesize that strain 4, which was isolated 20 days later, emerged from this population (i.e., was selected because of its resistance to NHS). The results of Southern hybridization of the strain-3 derivatives provide support for this hypothesis (figure 5B) and indicate DNA rearrangement as the mechanism underlying SLP expression and resistance to NHS. Thus, in addition to completely NHS-resistant and highly SLP-positive strains causing human infections (as seen in pairs 1, 3, and 4), a strain with an intermediate phenotype but with the capacity to rearrange into a more virulent phenotype also was pathogenic. In total, the results of analyses of these 4 pairs of strains associated with relapse point to at least 3 different mechanisms for relapse of clinical *C. fetus* infection: (1) rearrangement to up-regulate virulence, (2) rearrangement for antigenic variation, and (3) true latency of a fully virulent strain with later reactivation due to a change in clinical status.

**References**

37. Tu ZC, Dewhirst FE, Blaser MJ. Evidence that the Campylobacter fetus sap locus is an ancient genomic constituent with origins before mammals and reptiles diverged. Infect Immun 2001;69:2237–44.