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Comparison of the Nucleotide and Deduced Amino Acid Sequences of the S Genes Specified by Virulent and Avirulent Strains of Bovine Coronaviruses

XUMING ZHANG, KONSTANTIN G. KOUSOULAS, AND JOHANNES STORZ[1]

Department of Veterinary Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803

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The entire nucleotide sequences of the spike glycoprotein (S) genes of the highly virulent bovine coronavirus (BCV) strain BCV-LY138, the avirulent BCV-L9 and related Norden Vaccine (BCV-Vaccine) strains were determined using the polymerase chain reaction (PCR) to amplify cDNAs obtained by reverse transcription of viral RNA, and to produce single strand cDNAs for DNA sequencing. The S gene sequences of these viral strains were compared with those of recently published strains BCV-Mebus, BCV-Quebec, and BCV-F15. An open reading frame of 4092 nucleotides, encoding a protein of 1363 amino acid residues, was found in all six strains. Frameshifts and insertions or deletions were not observed except for the BCV-F15. The S gene sequences were more than 98% conserved overall inspite of different origins of the six viruses. There were 45 to 56 nt differences between the virulent and avirulent groups while there were 6 to 14 nt differences among four avirulent strains. Comparison of the deduced amino acid sequences indicated that the S proteins had typical properties of membrane glycoproteins. Nineteen N-linked glycosylation sites were predicted in five strains, and 18 of them were conserved in the avirulent strain BCV-L9. The sequence KRRSRR at the predicted proteolytic cleavage site was identified in five strains while the sequence KRRSVR was found in BCV-F15. Substitutions of few amino acids in the putative fusogenic domains and two prolines at 507 and 567 in the antigenic domains may cause altered immunogenic and other functional properties of the S proteins specified by the virulent and avirulent BCV strains. Nine amino acid substitutions between the virulent and avirulent groups may correlate with BCV virulence.

Bovine coronavirus (BCV) is a member of coronaviridae, causing severe diarrhea in newborn calves and winter dysentery in adult cattle (*1, 2*). It possesses a single-stranded, nonsegmented RNA genome with positive polarity (*3*). The virion contains four major structural proteins: the nucleocapsid protein (N), the transmembrane protein (M), the hemagglutinin/esterase protein (HE) and the spike protein (S) (*4*). The S glycoprotein is a predominant peplomeric structure forming the typical coronavirus morphology. It is synthesized as a high molecular weight (mol. wt.) precursor (gp190) which is cleaved to yield two comigrating subunit polypeptides: the N-terminal half (S1) and the C-terminal half (S2) with an approximate mol. wt. of 100 kDa (*3, 5*). The S glycoprotein functions in virus attachment to permissive cells, virus-induced cell fusion, elicitation of neutralizing antibodies and cell-mediated immunity (*6*). Two antigenic domains responsible for neutralization of BCV-Quebec and BCV-L9 have been identified on the S protein (Ref. (*7*); Hussain *et al.*, submitted). However, the location of the neutralizing epitopes on the BCV S protein has not yet been determined. Comparison of the S proteins with S-specific monoclonal antibodies (MAbs) revealed that different neutralizing and non-neutralizing epitopes as well as conformational epitopes were present in the S glycoproteins specified by the virulent and avirulent strains (Hussain *et al.*, submitted). In order to understand the molecular basis of the observed antigenic diversity of the S glycoproteins we successfully amplified, directly sequenced, and cloned the entire S genes of different BCV strains using the polymerase chain reaction (PCR). We report here the sequence characteristics of one virulent and two avirulent BCV strains and their comparison with the recently published sequences of three other BCV strains.

The strain BCV-L9 was derived from BCV-Mebus and passaged through different nonpolarized and highly polarized cells over 80 passages (*8*). The Norden Vaccine strain (BCV-Vaccine) was also derived from BCV-Mebus and used as vaccine by Norden Laboratories (Omaha, NE) (*1, 9*). The highly virulent wild-type strain BCV-LY138 was isolated from diarrheal fluid of a diseased calf in 1965 in Utah/USA (*10*), and maintained in calves through oral inoculation since then (*8, 11*). This strain replicates only in HRT-18 cells but not in numerous bovine cells (*8*). All strains were propagated in HRT-18 cells as described previously (*8*). After 24 h.p.i., cells were washed twice with phos-

[1] To whom reprint requests should be addressed.

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phate buffered saline (PBS), and RNA was isolated using isothiocyanate/cesium chloride gradients according to the method described previously (*12*). As control, RNA was isolated from uninfected cells.

The first-strand cDNA synthesis was carried out in a volume of 25 μl containing 50 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* DTT, 1 m*M* each of the four dNTPs, 25 U RNAsin (Bethesda Research Laboratories (BRL)), 2′ μ*M* 3′ primer, 1 μg total RNA, 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (BRL) for 1 hr at 42°, and the reaction was stopped by heating samples to 95° for 10 min and then cooled on ice. The RNA was denatured after adding 20 m*M* methylmercuric hydroxide (MeHgOH) and 70 m*M* 2-mercaptoethanol for 7 and 5 min at room temperature, respectively, incubated with oligonucleotide primer at 65° for 2 min, and then chilled on ice before adding other reagents. Two S gene specific primers were designed both for cDNA synthesis and PCR amplification according to the published sequence (*13*). The A3′ primer is antisense representing the sequence at the positions 2165–2187 (5′-*TTTGGATCC*AGGTTGCAGCTGTCGTGAAAGA-3′). The B3′ primer corresponds to the sequence downstream of the S gene (5′-*AACGGATCC*AATATATCGTCAGGAGCCAATA-3′). Both primers contain an extra *Bam*HI linker in the 5′ end for cloning purposes. The primers were synthesized in the Gene Assembler (Pharmacia-LKB, Piscataway, NJ) in our laboratory according to the manufacturer's instructions, and purified using Poly-PAK (Glen Research, Herndon, VA) or Oligo-PAK column (Millipore, Burlington, MA).

The reaction of second strand cDNA synthesis and amplification was carried out in PCR (MicroCycler, Eppendorf Inc., Fremont, CA) with 5 μl of the reverse transcription reaction in a total volume of 100 μl containing 20 m*M* Tris–HCl (pH 8.3), 25 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% Tween 20, 10 μg of bovine serum albumin (BSA), 2.5 U of *Taq* DNA polymerase (BRL), 200 μ*M* each of the four dNTPs, and 20 pmol each of the primers. The sense A5′ primer corresponds to the sequence upstream of the S gene (5′-*GCTGAATTC*GATAATGGTACTAGGCTGCATGAT-3′), and the B5′ primer represents the sequence at the positions 2165–2187 (5′-*GCTGAATTC*TCTTTCACGACAGCTGCAACCT-3′). These primers contain an extra *Eco*RI linker on the 5′ end as indicated. Each cycle consisted of a 1-sec denaturation at 96°, followed by 30-sec annealing (at 59°) and 2-min extention (at 72°) steps. After 30 to 35 cycles, the final products were extended for 7 min at 72°. Single-strand cDNA fragments were generated in a second PCR for sequencing. Briefly, one-tenth of the double stranded PCR product and a single primer with 40 pmol were used. Other reagents were the same as in the previous PCR. The cycle profile of 20 to 25 cycles was: 1-sec at 96°, 30-sec at 50°, 1-sec at 60°, 2-min at 72° (Fig. 1).

We sequenced the entire S genes of one virulent and two avirulent BCV strains which display distinct biological properties and epitopes as defined by MAbs to the reference strain BCV-L9. With the exception of 40 nt on the 5′-end and 50 nt on the 3′-end, the S gene sequences were determined in both directions at least once, as illustrated in Fig. 1. These sequences were compared with the recently published sequences for the strains BCV-Mebus (*14*), BCV-Quebec (*15*), and BCV-F15 (*13*), and their alignment is presented in Fig. 2 (origins of these strains are described in the legend). All of the S genes contained an ORF of 4092 nucleotides. The only variations among these sequences consisted of nucleotide substitutions. Frameshift, deletion, or insertion, and nonsense mutations were not observed except for BCV-F15. The few nucleotide substitutions (91 nt) represented approximately 2% of the sequence, and they seemed to be distributed randomly. By comparison with other strains, the following differences were detected in two regions in BCV-F15: The deletion of a base (T) at nucleotide 2316 resulted in a frameshift for 2 aa followed by the insertion of an A at nucleotide 2320 which returned the amino acid sequence to a homologous alignment (at aa position 772 within the cleavage site; also see Fig. 3); at the positions 3271–3272, the sequence GC was converted to CG. The intergenic and internal consensus sequence CYAAAC was conserved in all BCV strains. The two

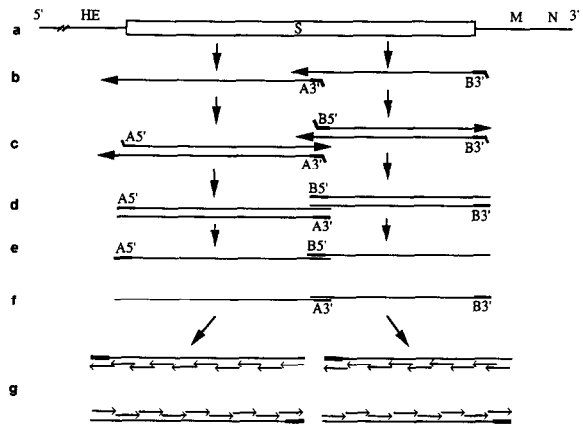

FIG. 1. Strategies of cDNA synthesis and DNA sequencing. The relative locations of S, M and N genes in the BCV genome (a); primer design and first-strand cDNA synthesis by reverse transcription (b); second-strand, PCR-mediated DNA synthesis (c); double-stranded DNA amplification by PCR (d); single-stranded DNA synthesis by PCR using an excess of 5′ primer (e) and 3′ primer (f); DNA sequencing strategies (g). A5′ and B5′ indicate two 5′ primers containing an extra *Eco*RI linker; A3′ and B3′ indicate two 3′ primers containing an extra *Bam*HI linker.

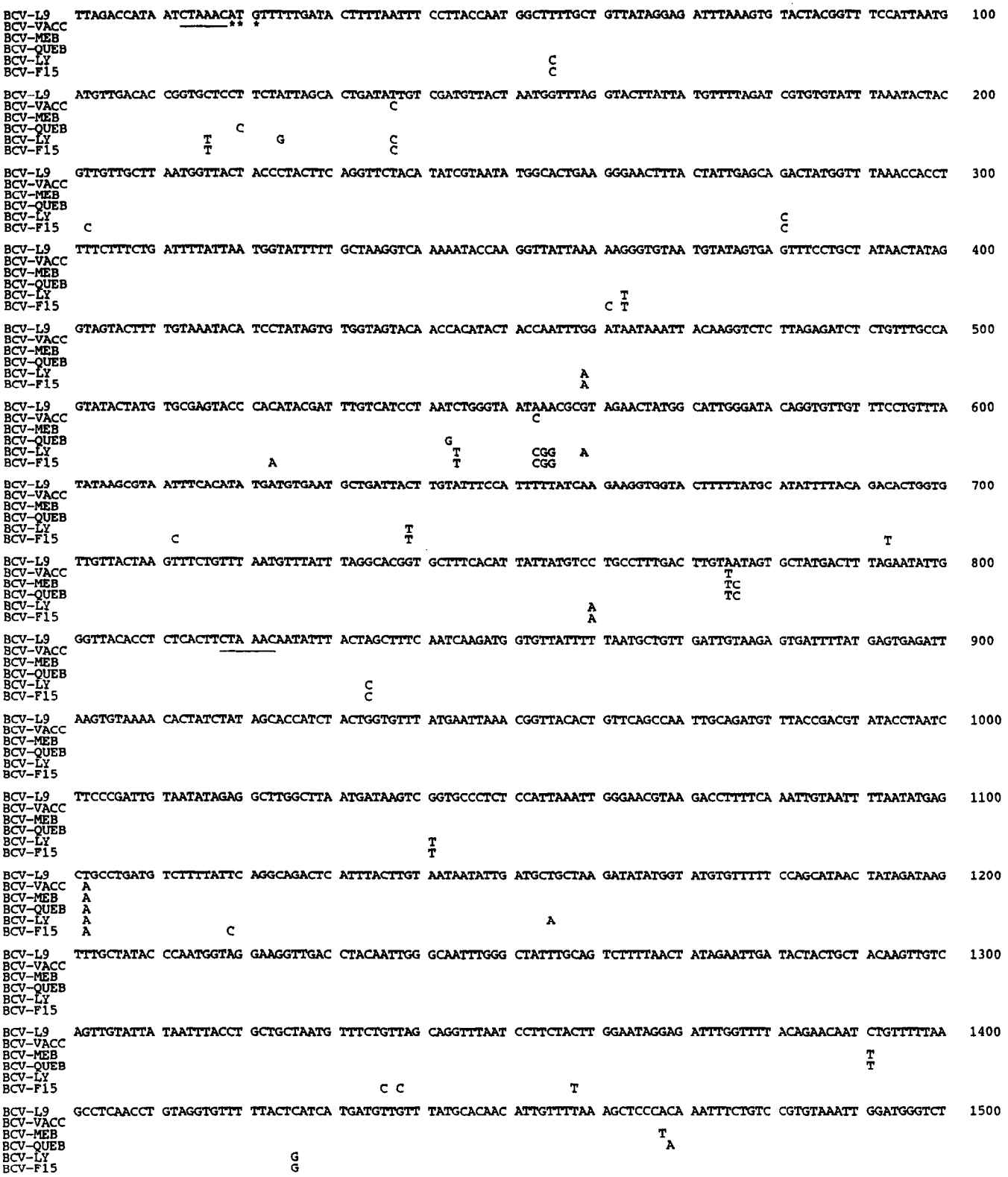

FIG. 2. Nucleotide sequence comparisons of the S genes of six BCV strains. DNA sequencing was carried out with the modified dideoxynucleotide chain termination procedure (*24*) using Sequenase (U.S.B., Cleveland, OH). Sequences were analyzed with the aid of the Sequence Analysis Software Package of the Genetics Computer Group of the University of Wisconsin and the MacVector Software (IBI, New Haven, CT). The S gene sequences of BCV-Mebus, BCV-Quebec, and BCV-F15 are obtained from Refs. (*14*), (*15*), and (*13*), respectively. The origins and properties of these strains are as follows: The strain BCV-Mebus was isolated from a calf with enteritis, and adapted to bovine fetal kidney cells described previously (*25*). The strain BCV-Quebec was isolated from a calf with enteritis by cultivation in Vero and MDBK cells in Quebec in 1979. Its biological properties were similar to BCV-Mebus (*26*). The wild-type strain BCV-F15 was isolated in HRT cells from a calf with enteritis in France in 1979 (*27*). The consensus sequences are underlined, and the start and stop codes are marked by asterisks.

```
BCV-L9    TTGTGTGTAG GTAATGGTCC TGGTATAGAT GCTGGTTATA AAAATAGTGG TATAGGCACT TGTCCTGCAG GTACTAATTA TTTAACTTGC CATAATGCTG  1600
BCV-VACC
BCV-MEB
BCV-QUEB
BCV-LY                  G    T                                                                             T
BCV-F15                                                                                 C

BCV-L9    CCCAATGTGA TTGTTTGTGC ACTCCCGACC CCATTACATC TAAATCTACA GGGCCTTACA AGTGCCCCCA AACTAAATAC TTAGTTGGCA TAGGTGAGCA  1700
BCV-VACC
BCV-MEB           A
BCV-QUEB
BCV-LY                               A                                   T                      T
BCV-F15           A                  A

BCV-L9    CTGTTCGGGT CTTGCTATTA AAAGTGATTA TTGTGGAGGT AATCCTTGTA CTTGCCAACC ACAAGCATTT TTGGGTTGGT CTGTTGACTC TTGTTTACAA  1800
BCV-VACC
BCV-MEB                                                                                   C
BCV-QUEB
BCV-LY                                                                                                 T
BCV-F15

BCV-L9    GGGGATAGGT GTAATATTTT TGCTAATTTT ATTTTGCATG ATGTTAATAG TGGTACTACT TGTTCTACTG ATTTACAAAA ATCAAACACA GACATAATTC  1900
BCV-VACC
BCV-MEB
BCV-QUEB                                        T
BCV-LY                      C
BCV-F15

BCV-L9    TTGGTGTTTG TGTTAATTAT GATCTTTATG GTATTACAGG CCAAGGTATT TTTGTTGAGG TTAATGCGCC TTATTATAAT AGTTGGCAGA ACCTTTTATA  2000
BCV-VACC                                                                            A
BCV-MEB                                                                             A
BCV-QUEB                                                                            A
BCV-LY                                                                              A
BCV-F15                                           T                         C       A

BCV-L9    TGATTCTAAT GGTAATCTCT ATGGTTTTAG AGACTACTTA ACAAACAGAA CTTTTATGAT TCGTAGTTGC TATAGCGGTC GTGTTTCAGC GGCCTTTCAT  2100
BCV-VACC
BCV-MEB
BCV-QUEB
BCV-LY
BCV-F15

BCV-L9    GCTAACTCTT CCGAACCAGC ATTGCTATTT CGGAATATTA AATGCAATTA CGTTTTTAAT AATACTCTTT CACGACAGCT GCAACCTATT AACTATTTTG  2200
BCV-VACC
BCV-MEB
BCV-QUEB
BCV-LY         T
BCV-F15                        C

BCV-L9    ATAGTTATCT TGGTTGTGTT GTCAATGCTG ATAATAGTAC TTCTAGTGTT GTTCAAACAT GTGATCTCAC AGTAGGTAGT GGTTACTGTG TGGATTACTC  2300
BCV-VACC
BCV-MEB
BCV-QUEB
BCV-LY                                                        C
BCV-F15       C                                        G      C

BCV-L9    TACAAAAAGA CGAAGTCGTA GAGCGATTAC CACTGGTTAT CGGTTTACTA ATTTTGAGCC ATTTACTGTT AATTCAGTAA ATGATAGTTT AGAACCTGTA  2400
BCV-VACC
BCV-MEB                                                          C
BCV-QUEB
BCV-LY
BCV-F15                   CGTA

BCV-L9    GGTGGTTTGT ATGAAATTCA AATACCTTCA GAGTTTACTA TAGGTAATAT GGAGGAGTTT ATTCAAACAA GCTCTCCTAA AGTTACTATT GATTGTTCTG  2500
BCV-VACC
BCV-MEB
BCV-QUEB
BCV-LY                                                                             T
BCV-F15                                      A

BCV-L9    CTTTTGTCTG TGGTGATTAT GCAGCATGTA AATCACAGTT GGTTGAATAT GGTAGCTTCT GTGACAATAT TAATGCTATA CTCACAGAAG TAAATGAACT  2600
BCV-VACC
BCV-MEB
BCV-QUEB
BCV-LY                                                                  T
BCV-F15                      G                                          T

BCV-L9    ACTTGACACT ACACAGTTGC AAGTAGCTAA TAGTTTAATG AATGGTGTCA CTCTTAGCAC TAAGCTTAAA GATGGCGTTA ATTTCAATGT AGACGACATC  2700
BCV-VACC
BCV-MEB
BCV-QUEB
BCV-LY
BCV-F15

BCV-L9    AATTTTTCCC CTGTATTAGG TTGTTTAGGA AGCGATTGTA ATAAAGTTTC CAGCAGATCT GCTATAGAGG ATTTACTTTT TTCTAAAGTA AAGTTATCTG  2800
BCV-VACC
BCV-MEB
BCV-QUEB                                       C
BCV-LY                                                                    T
BCV-F15                                         A                   T

BCV-L9    ATGTCGGTTT CGTTGAGGCT TATAATAATT GTACTGGAGG TGCCGAAATT AGGGACCTCA TTTGTGTGCA AAGTTATAAT GGTATCAAAG TGTTGCCTCC  2900
BCV-VACC
BCV-MEB
BCV-QUEB
BCV-LY               T
BCV-F15                                           G            C                C

BCV-L9    ACTGCTCTCA GTAAATCAGA TCAGTGGATA CACTTTGGCT GCCACCTCTG CTAGTCTGTT TCCTCCTTGG TCAGCAGCAG CAGGTGTACC ATTTTATTTA  3000
BCV-VACC
BCV-MEB                                                                            T             T
BCV-QUEB                                                                           T             T
BCV-LY       A        A                                                                              C
BCV-F15               A                                  T
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FIG. 2—*Continued*

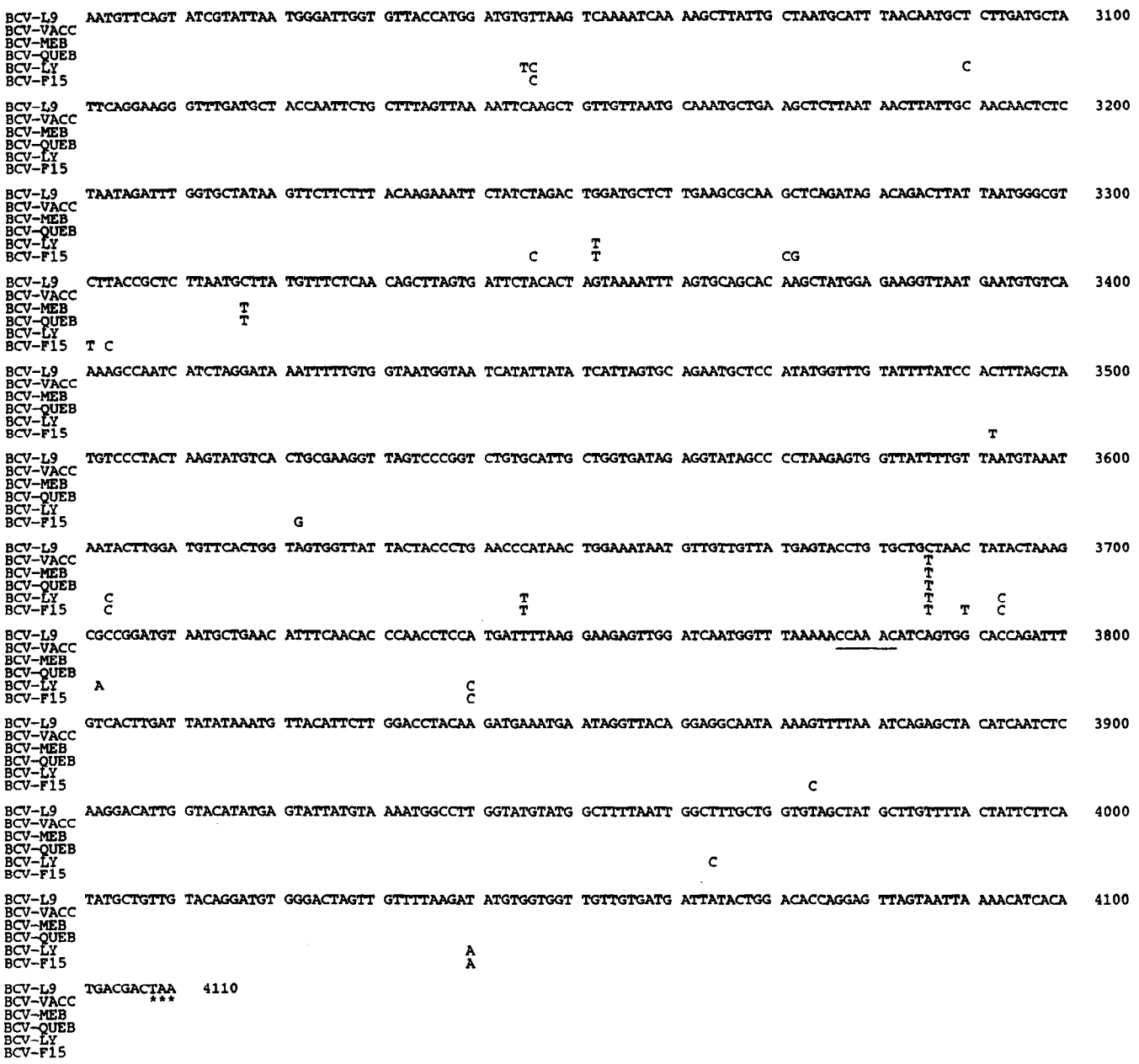

FIG. 2—*Continued*

most similar sequences were those of BCV-L9 and BCV-Vaccine (6 nt differences) in paired comparisons, and the most distant were those of BCV-Quebec and BCV-F15 (74 nt differences). The virulent wild-type strain BCV-LY138 was evolutionally distant from the avirulent strains (total number of differences between 45 to 56 nt). The French strain BCV-F15 also differed from the avirulent strains (total number of differences between 55 to 74 nt). Although there were 55 nt differences between BCV-LY138 and BCV-F15, the same nucleotide substitutions occurred in 29 nt.

An alignment of the deduced amino acid sequences of the S proteins of the six BCV strains is shown in Fig. 3. All six S genes encoded a predicted protein of 1363 aa residues, having a mol. wt. of approximately 150 kDa. Nineteen potential N-linked glycosylation sites were predicted in 5 strains while 18 of them were conserved in BCV-L9. Fifty-seven cysteine residues were found in BCV-L9 while 56 of them were conserved in the other 5 strains. The BCV-F15 had an additional cysteine at position 840. The glycosylated protein had an estimated mol. wt. of approximately 190 kDa. All six S proteins possessed at least 98% sequence identity in paired comparisons. As with the nucleotide se-

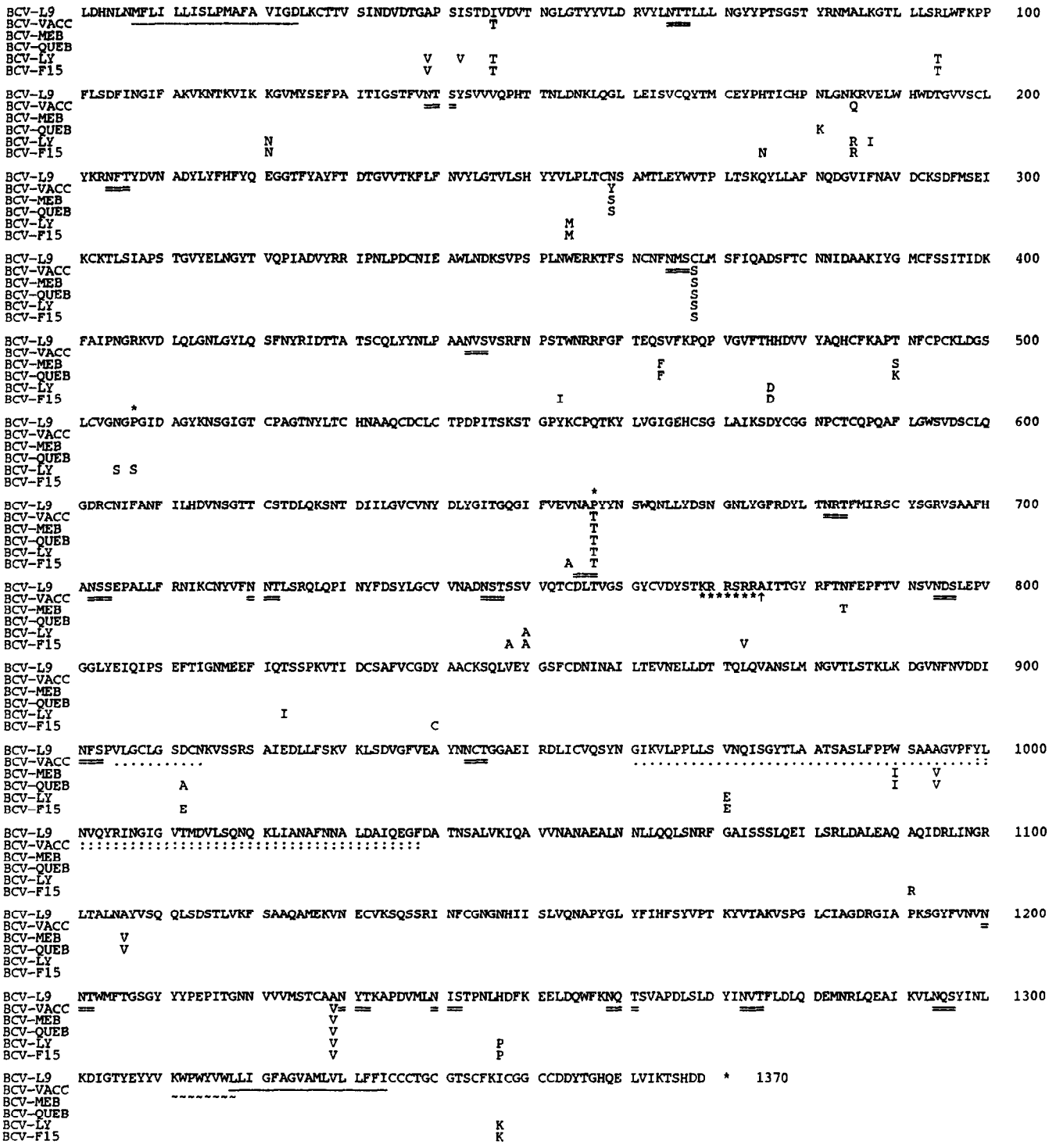

FIG. 3. Amino acid sequence comparisons of the predicted S proteins of six BCV strains. The predicted signal peptide and intramembrane anchoring sequences are underlined. The predicted cleavage site is indicated by an arrow, and its sequence is marked by asterisks underneath. The 19 predicted N-linked glycosylation sites are double underlined. The conserved sequence is marked by (~). The putative hydrophobic domains are indicated by (.), and the adjacent heptad repeat sequences by (:). Two proline substitutions are indicated by asterisks on the top.

quences, the most related proteins were those of BCV-Mebus and BCV-Quebec (4 aa differences) or BCV-L9 and BCV-Vaccine (6 aa differences); the most divergent proteins were those of BCV-Mebus and BCV-F15 or BCV-Quebec and BCV-F15 (26 aa differences, respectively). The alignment reveals that the strain BCV-

Quebec is closely related to the avirulent strains BCV-Mebus, BCV-L9, and BCV-Vaccine (6 to 14 nt and 4 to 11 aa differences, respectively), suggesting that this strain is a variant of the prototype BCV-Mebus. In contrast to the nucleotide sequences, the amino acid sequences of BCV-LY138 and BCV-F15 were more homologous (13 aa differences). As shown in Fig. 3, 9 amino acids in the avirulent strains were substituted in both BCV-LY138 and BCV-F15: A to V (38), R to T (94), K to N (121), L to M (254), H to D (476), V to A (749), V to E (980), A to V (1106), H to P (1247), and I to K (1347). This suggests that the wild-type strain BCV-F15 is possibly a virulent strain, and that the 9 amino acids, especially the proline (at 1247) and lysine (at 1347) on the carboxy-terminal, may relate to BCV virulence. Amino acid differences between the virulent and avirulent groups varied from 16 to 26 aa. Apparently, most of these substitutions occurred in the S1 peptide (Fig. 3).

Comparisons of the amino acid sequences in the antigenic region (*14, 16*) suggested that two proline substitutions may cause the antigenic differences: at amino acid position 507, a proline in the avirulent strain BCV-L9 was substituted by a serine in the virulent strain BCV-LY138, and another proline (at 567) was substituted by a threonine in all other strains. Proline is considered a helix-breaking residue, which may influence significantly the conformation and secondary structure of peptide molecules (*17*). The proline substitutions resulted in decreased hydrophilicity, surface probability, and antigenicity, but increased flexibility of the peptide in this region (data not shown). Our previous study indicated that one neutralizing epitope of BCV-L9 was present in BCV-Vaccine but absent in the virulent strain BCV-LY138, and one non-neutralizing epitope was present only in the avirulent strain BCV-L9 (Hussain *et al.,* submitted). The number of proline substitutions in this region coincides with the number of conformational epitopes absent in BCV-LY138 and BCV-Vaccine lead us to conclude that substitutions of these prolines to other amino acids may alter the conformation of the S protein, and cause the loss of conformation-dependent epitopes in BCV-LY138 and BVC-Vaccine. This hypothesis is currently under investigation. It is unclear whether substitutions of these prolines and other amino acids in this region also correlate with BCV virulence.

Our previous studies revealed that the biological properties of fusion, plaque formation and host cell range were evidently different between the virulent and avirulent groups of BCV strains (*8*). The fusion activity of BCV is believed to be associated with the S polypeptide and the cleavage of the S into S1 and S2 subunits (*5, 18*). The sequence KRRSRR at the predicted proteolytic cleavage site was conserved in the virulent strain BCV-LY138 and in the avirulent strains while the sequence KRRSVR occurred in the wild-type BCV-F15 (Fig. 3). The cleavage site of BCV S protein is located in a hydrophilic area, in contrast to paramyxoviruses and myxoviruses, in which the cleavage site is located in hydrophobic domains (20–30 aa) of the fusion proteins (*19, 20*). These observations imply that there might be a different mechanism involved in BCV-induced cell fusion. Parker *et al.* (*14*) indicated that a sequence VLGCLGSAC (905–913) on the S protein of BCV-Quebec may constitute a portion of the fusogenic domain within the BCV S2 subunit. This stretch is similar to the sequence LLGCIGSTC of MHV-A59, which contains a neutralizing epitope (*25*). By comparing with paramyxovirus and retrovirus, Chambers *et al.* (*26*) proposed that the hydrophobic region adjacent to heptad repeat sequences may be the potentially fusion-related domain in the S proteins of mouse hepatitis virus, infectious bronchitis virus, and transmissible gastroenteritis virus. After multiple alignments it is found that the heptad repeat sequences are located in the S2 at positions 999–1038 which are conserved in all strains, and the hydrophobic regions are located at 961–998 (see Fig. 3). However, a direct involvement of these sequences in BCV fusion has yet to be demonstrated. Similarly, the E1 protein of Semliki Forest virus (SFV) does not contain a hydrophobic N-terminal region. It has been proposed, however, that in E1 an internal uncharged stretch of 17 residues, located about 80 aa from the N-terminal, might act as the putative fusogenic domain (*27*). Interestingly, we found three patterns of amino acid substitution in the putative fusogenic domain: at position 971, V in the avirulent strains was substituted by E in the virulent strains; W (898) and A (993) were substituted by I and V in BCV-Mebus and BCV-Quebec, respectively. It is unclear, however, whether these amino acid changes in the predicted cleavage sites and the putative fusogenic domains reflect any functional differences, such as fusion activities among different BCV strains.

ACKNOWLEDGMENTS

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