Immunological Markers of the R4 Protein of *Streptococcus agalactiae*

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Received 7 April 2005/Returned for modification 11 July 2005/Accepted 25 August 2005

This study focuses on immunological markers of R4, an important *Streptococcus* group B (GBS) protein. The results obtained by using rabbit antisera and purified proteins for antigens in enzyme-linked immunosorbent assay-based experiments provided evidence that R4 possesses two antigenic determinants. One of the determinants is shared with the alpha-like protein 3 (Alp3) of GBS, was named R4/Alp3 common, and was expressed by GBS, which possessed the Alp3-encoding gene *alp3* or the R4-encoding gene *rib*. The other antigenic determinant was detected only in *rib*-positive GBS organisms and was named R4 specific. This determinant probably is an immunological marker unique to the R4 protein. Neither of the antigenic R4 determinants showed serological cross-reactivity with the GBS proteins Cα, Cβ, and R3 or with alpha-like protein 2. Of 60 clinical serotype III GBS strains, 56 (93%) isolates possessed the *rib* gene and 50 (89%) of the *rib*-positive isolates expressed levels of R4 detectable by antibody-based tests, consistent with R4 expression failure or low-level expression in ~10% of *rib*-positive GBS. *alp3* was not detected in type III GBS but was possessed by six of eight type V strains and six of six type VIII strains. All *alp3*-positive strains were recognized by the R4/Alp3 common antibodies, but none of them were recognized by the R4-specific antibodies. NCTC 9828, a reference strain for R3 and R4, expressed the determinant R4/Alp3 common but not R4 specific. A monoclonal R4 antibody, previously considered to be R4 specific and used in GBS serotyping, targeted R4/Alp3 common and is thus not R4 specific. The results show that failure to discriminate between R4 specific and R4/Alp3 common by antisera designed for GBS serotyping can result in the false identification of Alp3 as R4 or vice versa, whereas anti-R4 antibodies targeting only the determinant R4 specific will detect only R4. Both R4 and Alp3 need further evaluation with respect to the immunobiological function of each distinct antigenic determinant, for instance, with regard to their potential as GBS vaccine components.

*Streptococcus agalactiae* (group B streptococci [GBS]) is an important cause of infections in humans, notably in neonates. Serotyping based on the capsular polysaccharide antigens Iα, Iβ, and II through VIII has been used extensively in epidemiological classification of GBS, sometimes supplemented by serotyping on the basis of strain-variable and surface-anchored protein antigens. These proteins include the C proteins Cα (encoded by *bec* [22]) and Cβ (encoded by *bac* [10]) and the classical R proteins R1, R3, and R4 (8, 18, 35). More recently, protein Rib was described (33), but this protein seems to be identical to the classical R4 protein (1, 30). Alpha-like proteins described recently, Alp2 (encoded by *alp2* [16]) and Alp3 (encoded by *alp3* [16]), may be variants of the classical R1 protein (19; J. Maeland and R. Valsoe Lyng, Abstr. 13th European Congress of Clinical Microbiology and Infectious Diseases, abstr. P611, 2003). These proteins, except for Cβ, belong to a protein family characterized among other things by similarity in primary structure, with up to 100% homology for some of the protein stretches (16, 34), and by their generation of ladder-like patterns on Western blots, probably due to large and identical repeat units which vary in number from strain to strain (9, 22, 34). Horizontal transfer of genetic elements between strains followed by recombinational events has been advocated as an explanation of the structural relatedness and mosaicism of these proteins (16). These proteins may be important virulence factors in GBS, and they elicit antibodies which are protective in animal models (17, 21, 26, 31, 32, 33). Some of the proteins show serological cross-reactivity (17, 19, 31, 32) attributed to structural matching, and this reactivity may hamper the reliability of antibody-based protein detection, for instance, in GBS serotyping. Genotyping instead of serotyping has become an approach to keep clear of this problem (4, 5, 11, 12, 13). Alternatively, or as a supplement to genotyping, it may be possible to increase the reliability of antibody-based GBS typing through better knowledge of the immunological features of the proteins.

In an earlier study from this laboratory, it was found that the alpha-like protein Alp3 possessed an antigenic determinant which was also possessed by the R4 protein and was called R4/Alp3 common by us (19). Alp3 also possessed an antigenic determinant which was shared with Alp2 and was named Alp2/Alp3 common (19). PCR results have indicated frequent expression of the Alp3 and R4 proteins (13), and R4 is also known to be frequently expressed on the basis of antibody-based tests (15, 24, 32), meaning a high frequency of expression by GBS strains of the antigenic R4/Alp3 common determinant. Thus, the reliability of R4 detection by antibody-based methods could be seriously hampered by antibodies targeting the Alp3/R4 common determinant, unless the cross-reacting antibodies have been eliminated. On the other hand, reliable antibody-based detection of R4 requires that this protein harbors one or more R4-specific immunological markers. Considerations along these lines encouraged the present study of immunological markers of the R4 protein of GBS.
### MATERIALS AND METHODS

#### Bacterial strains.

The GBS reference and prototype strains used in this study were those listed in Table 2, seven additional strains which have been described in previous reports (14, 26), and the type VIII strain JM9 (17). These strains included at least one isolate of each of the nine capsular antigen types of GBS, and strains which expressed at least one of the well-defined, strain-variable, and surface-localized GBS proteins. The strains 64/95 (V/Ralp3) and 65604 (III/Ralp4), our prototypes for the proteins Alp3 and R4, were used for the generation of antisera and for the preparation of the Alp3 and R4 proteins. Clinical GBS strains were type III, V, and VIII isolates arbitrarily selected from our strain collection. These isolates were from patients hospitalized with invasive GBS disease in various Norwegian hospitals during the last 12 years and had been forwarded to our laboratory for serotyping. All isolates included in this study were tested by PCR for possession of the genes alp2, alp3, and rib, encoding the proteins Alp2, Alp3, and R4, respectively. The bacteria were cultured on blood agar plates or in Todd-Hewitt broth as described elsewhere (3).

#### Antiseria.

Antiseria were raised against whole cells of GBS or against the purified R4 protein as described previously (1, 2). A murine monoclonal antibody (MAb), the immunoglobulin G (IgG) F39, raised against the R4 protein (R4 MAb) was that described previously (1). This antibody was used after incubation with whole GBS cells to remove non-specific binding before absorption with GBS whole cells (27), by immunoblotting, by negative indirect enzyme-linked immunosorbent assay (ELISA) with antisera against GBS strains with no expression of the protein of interest, and by a negative test for capsular polysaccharide antibodies by Western blotting. Western blotting was performed as previously described in detail (25), using material (10 µl) solubilized with hot dodecyl sulfate from whole cells of GBS, applied to 10% (wt/vol) polyacrylamide separating gels, and, after electrophoresis, transferred to polyvinylidene difluoride membranes (Bio-Rad). Probing was done against antisera diluted 1:400. Antibody binding was detected using horseradish-conjugated anti-immunoglobulin (1:10,000). Strips containing standard proteins were stained with amido black.

#### Oligonucleotide primers.

 Primer pairs for the genes alp2, alp3, alp (alp2 plus alp3), and rib were constructed (Eurogentec S.A., Liege, Belgium) according to recommended specifications (13) and as described previously (19) and were as follows: the pair bal2S1-balA2 for alp2 (GenBank accession no. AF208158), with an amplicon length of 426 bp; the pair bal2S1-bal3A for alp3 (GenBank accession no. AF245663), with an amplicon length of 321 bp; the pair balS1-balA for alp2 plus alp3, with an amplicon length of 446 bp; and the pair ribS2-ribA2 for rib (GenBank accession no. U58333), with an amplicon length of 225 bp. PCR. For all primer sets, PCR was performed as described earlier for detection of the Co-encoding gene bca (20), including detection of the PCR products by electrophoresis in 2% (wt/vol) agarose gels. The performance of the PCRs was evaluated by us in a recent study, in which the same primer pairs were used (19). Sequence analysis. PCR products were purified by using the QIAquick PCR purification kit (Qiagen). The products were sequenced directly on an ABI 373 DNA sequencer using an ABI PRISM dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems). Alignment analysis of the sequences was performed using the program Sequence Navigator (PE Applied Biosystems).

#### RESULTS AND DISCUSSION

### Antigenic R4 determinants examined by polyclonal antisera.

Rabbit antiserum against whole cells of the GBS strain 65604 (III/Ralp4) and antiserum against purified R4 from the same strain showed cross-reactivity with Alp3 in addition to the reactivity against R4 (Table 1). After removal by absorption of the antibodies targeting Alp3, both antisera still recognized R4. These results indicate that rabbits immunized with R4 raised antibodies against an antigenic determinant shared by Alp3 and R4, provisionally called R4 specific antibodies, whereas both the antisera still recognized R4. These results indicate that rabbits immunized with R4 raised antibodies against an antigenic determinant shared by Alp3 and R4, provisionally called R4 specific antibodies, whereas both the antisera still recognized R4.

#### Fluorescent-antibody test (FAT).

A whole-cell-based indirect immunofluorescence assay was performed as described elsewhere (2). The fluorescent was graded from 0 to 3+, with scores of 2+ and 3+ indicative of a positive test. All primary antisera were used in a dilution of 1:40, which generated 3+ reactions with positive-control isolates and negative test results with negative-control strains when commercial fluorescent anti-IgG conjugates were used as recommended by the manufacturer (Dako Cytomation).

#### Western blotting.

Western blotting was performed as previously described in detail (25), using material (10 µl) solubilized with hot dodecyl sulfate from whole cells of GBS, applied to 10% (wt/vol) polyacrylamide separating gels, and, after electrophoresis, transferred to polyvinylidene difluoride membranes (Bio-Rad). Probing was done against antisera diluted 1:400. Antibody binding was detected using horseradish-conjugated anti-immunoglobulin (1:10,000). Strips containing standard proteins were stained with amido black.

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| Strain against which antigen was raised | Titer against coating antigen: |
|----------------------------------------|-------------------------------|
| R4                                     |                              |
| Alp3                                   |                              |
| 65604 (III/Ralp4), whole cells          |                               |
| Unabsorbed                             | >25,600                       |
| Absorbed 64/95 (V/Ralp3)                | 6,400                         |
| Absorbed 65604 (III/Ralp4)              | 200                           |
| 65604 R4, purified                      |                               |
| Unabsorbed                             | 6,400                         |
| Absorbed 64/95 (V/Ralp3)                | 3,200                         |
| Absorbed 65604 (III/Ralp4)              | 200                           |
TABLE 1. Cross-reactivities of the antigenic determinants R4 specific and R4/Alp3 common with reference and prototype GBS strains, tested by the absorption ELISA

| Strain tested for cross-reactivity | OD₄₀₀ reduction (%) with antibodies against* | R4 specific | R4/Alp3 common |
|-----------------------------------|--------------------------------------------|-------------|----------------|
| NCTC 12906 (335; Ia/Cα)           |                                            | 0           | 0              |
| 15626 (IV/Cβ)                     |                                            | 0           | 0              |
| ATCC 12403 (D136C; IIIR1alp2)      |                                            | 0           | 100            |
| 64/95 (V/R1alp2)                  |                                            | 0           | 0              |
| ATCC 49447 (10/84; V/R3)          |                                            | 100         | 100            |
| 65604 (III/R4rib)                 |                                            | 100         | 98             |
| BM 110 (III/R4rib)                |                                            | 0           | 0              |
| 5/92 (III)                        |                                            | 0           | 0              |

* The antigen-antibody combinations in the absorption ELISA were R4 for the coating antigen and antiserum to purified R4 cross-absorbed by whole cells of strain 64/95 (V/R1alp3) (R4 specific) and Alp3 for the coating antigen and unabsorbed anti-R4 serum (R4/Alp3 common), based on the results shown in Table 1.

R4 specific and R4/Alp3 common showed cross-reactivity with any of the proteins Cα, Cβ, Alp2, and R3 (Table 2).

Of the two antigenic R4 determinants, R4 specific has, to our knowledge, not been clearly defined previously. The existence of R4/Alp3 common was expected on the basis of earlier findings (19) and from the demonstration of serological cross-reactivity between R4 and the R28 protein of group A streptococci, which is almost identical to Alp3 of GBS (16, 31, 32). Since the N terminus of Rib/R4 showed less than 65% homology with the proteins Cα, Cβ, and Alp3 (16), the R4 specific site may be located towards this terminus, while the R4/Alp3 common determinant may be located in the repeats, since the Alp3 and R4 repeats were almost identical (16). However, at the present time, these suggestions are hints which need experimental confirmation, for instance, by epitope mapping. Also, we cannot exclude the possibility that R4 harbors additional antigenic determinants which could not be detected by the experiments performed in this study. Moreover, one or both of the R4 determinants may be immunologically heterogeneous; for instance, each of them may comprise several epitopes, each with a different antigenic specificity.

Performance of the anti-R4 antibodies in whole-cell-based immunofluorescence. Since whole-cell-based testing has frequently been used in GBS serotyping, we examined the performance and specificity of the R4 specific and R4/Alp3 common antibodies in a whole-cell-based fluorescent-antibody test, along with testing by PCR for the genes alp2, alp3, and rib, encoding Alp2, Alp3, and R4, respectively. This testing also enabled evaluation of the concordance between gene possession and gene expression. We found that the sequences of the PCR products generated from the strains 12403 (alp2), 64/95 (alp3), JM9 (alp3), and 65604 (rib) matched completely the corresponding extents of the sequenced genes and that the PCRs showed complete agreement with the known protein expression when our 15 reference and prototype GBS strains were examined. This confirms earlier findings regarding the specificity of the PCRs (13, 19). Figure 1 shows representative PCR results. In the FAT, the anti-R4 specific and anti-R4/Alp3 common sera, prepared by cross-absorption as shown in Table 3, recognized our reference and prototype GBS strains as identical to the antibody binding shown by these strains in the absorption ELISA. In indirect ELISA with extracted antigens, both antisera designed for the FAT recognized GBS proteins, as was expected from the data described above. These results substantiated the supposition that the specificities of the ELISA-reactive and the FAT-reactive antibodies were the same and also showed that both the R4 specific and R4/Alp3 common sites were available for antibody binding at the bacterial cell surfaces. A total of 74 clinical GBS strains of the serotypes III (n = 60), V (n = 8), and VIII (n = 6) were examined by the FAT and by the PCRs. The results are summarized in Table 3.

Of the 60 clinical serotype III strains tested, 56 (93%) isolates were rib positive, whereas rib was not detected among the type V and type VIII strains. Only rib-positive strains were recognized by the putative R4 specific antisera. alp3 predominated among the type V and type VIII strains but was not detected in any of the type III isolates. Both alp3- and rib-

TABLE 2. Cross-reactivities of the antigenic determinants R4 specific and R4/Alp3 common with reference and prototype GBS strains, tested by the absorption ELISA

| Strain tested for cross-reactivity | OD₄₀₀ reduction (%) with antibodies against* | R4 specific | R4/Alp3 common |
|-----------------------------------|--------------------------------------------|-------------|----------------|
| NCTC 12906 (335; Ia/Cα)           |                                            | 0           | 0              |
| 15626 (IV/Cβ)                     |                                            | 0           | 0              |
| ATCC 12403 (D136C; IIIR1alp2)      |                                            | 0           | 100            |
| 64/95 (V/R1alp2)                  |                                            | 0           | 0              |
| ATCC 49447 (10/84; V/R3)          |                                            | 100         | 100            |
| 65604 (III/R4rib)                 |                                            | 100         | 98             |
| BM 110 (III/R4rib)                |                                            | 0           | 0              |
| 5/92 (III)                        |                                            | 0           | 0              |

* The antigen-antibody combinations in the absorption ELISA were R4 for the coating antigen and antiserum to purified R4 cross-absorbed by whole cells of strain 64/95 (V/R1alp3) (R4 specific) and Alp3 for the coating antigen and unabsorbed anti-R4 serum (R4/Alp3 common), based on the results shown in Table 1.

FIG. 1. Results representative of PCR for the genes alp2 (lane1), alp3 (lane 2), and rib (lane 3) are shown. The strains 12403 (III/R1alp2; lane 1), JM9 (VIII/R1alp3; lane 2), and 65604 (III/R4rib; lane 3) were tested.

TABLE 3. Distribution of the R4 protein determinants R4 specific and R4/Alp3 common among serotype III, V, and VIII GBS strains, determined by a FAT, and of the genes alp2, alp3, and rib, determined by PCR

| GBS tested | No. of strains | No. of strains | R4 specific* | R4/Alp3 common* |
|------------|---------------|---------------|--------------|----------------|
| Serotype III strains | 60            | 47            | 50           |                |
| rib positive/alp negative | 56            | 47            | 50           |                |
| rib negative/alp2 positive | 2             | 0             | 0            |                |
| rib negative/alp positive | 2             | 0             | 0            |                |
| Serotype V strains | 8             | 0             | 6            |                |
| rib negative/alp3 positive | 6             | 0             | 6            |                |
| rib negative/alp2 positive | 1             | 0             | 0            |                |
| rib negative/alp negative | 1             | 0             | 0            |                |
| Serotype VIII strain | 6             | 0             | 6            |                |
| rib negative/alp3 positive | 6             | 0             | 6            |                |

* Anti-R4 serum cross-absorbed with strain 64/95 (V/R1alp3).

+ Antiserum against strain 64/95 (V/R1alp3) cross-absorbed with the strains 12403 (III/R1alp2) and 52/95 (V/Cβ, R3).

* One of the strains expressed the R3 protein.

+ The isolate expressed Cβ and R3.
positive strains were recognized by the R4/Alp3 common antiserum, as distinct from the recognition by the R4 specific serum. However, while all of the alp3-positive strains were FAT positive with the R4/Alp3 common antibodies, 9 of 56 rib-positive strains were FAT negative with the R4 specific antibodies, and of these, 6 isolates were also negative with the R4/Alp3 common antibodies. To further test if this could be due to no expression or low-level expression of R4, all nine isolates were examined by the absorption ELISA by using a bacterial density of ~10~10~ CFU ml~1~ for the absorption. All of these isolates showed either no antibody binding, i.e., no reduction of the ELISA signaling, or only minor antibody binding in tests with either category of the anti-R4 antibodies, compared to >95% reduction of signaling by both antibodies when five other GBS strains with strong signaling in the FAT were tested. A rib- and alp-negative type III strain (strain 5/92) caused no OD reduction. These results were corroborated by Western blotting, with representative results shown in Fig. 2A.

alp2 occurred among the isolates but rarely (Table 3). Antibody-based testing, as routinely done in our laboratory for GBS serotyping by the FAT (15, 24), showed that none of the 74 GBS strains examined expressed C~a~, 1 isolate expressed C~b~, and 2 strains expressed the R3 protein (Table 3). None of these strains harbored alp2, alp3, or rib. Taken together, the results show that 72 (97%) of the 74 GBS strains examined expressed a ladder-forming protein or possessed a gene(s) known to encode that type of protein. The results substantiate the specificity of the R4 specific antiserum in R4 protein detection, elucidate the consequences of the R4/Alp3 common antibodies in admixture with the R4 specific antibodies in antisera designated for GBS serotyping, and highlight the problem of discrepancy between gene possession and gene expression.

It is obvious from the results of this study that antibodies against R4/Alp3 common have to be removed in order to obtain sera specific for R4. Only recently has knowledge of the antigenic R4/Alp3 common determinant become available (16, 19, 31). Thus, antibodies against the R4/Alp3 common site may have been present in some of the typing sera which over the years have been used to identify R4, resulting in some erroneous findings and interpretations. For instance, in a study it was found that recognition of GBS strains by R protein antiserum correlated with the presence of either of the rib and alp3 genes (13), probably due to R4/Alp3 common antibodies present in the serum. In another study, 55.6% of type V GBS strains showed coexpression of R1 and R4 (7). This could have been an erroneous conclusion caused by R4/Alp3 common antibodies in an antiserum assumed to be R4 specific, since according to the results of another study (13) and to our own findings (Table 3), the great majority of type V GBS were alp3 PCR positive but were rib PCR negative. This interpretation also accords with a recent observation that type V GBS, with coexpression of R1 and R4 as determined by antibody-based methods, showed negative PCR and Southern blot results with primers and probes specific for the R4 gene (30). The same may be true for the prototype VIII strain JM9, said to be R4 positive (17). In our hands, this strain was alp3 PCR positive and was targeted by the R4/Alp3 common antibodies but was rib PCR negative and was not targeted by the R4 specific antibodies (not shown). Thus, some earlier results and interpretations need reconsideration in light of the role of the antigenic R4/Alp3 common determinant, which also affects work done in our laboratory.

The occurrence and distribution of the alp and rib genes in the GBS strain collection of Norwegian origin agreed well with the findings obtained with Australasian GBS strains (13) and also agreed with the prevalence of R4 expression by isolates in different type III GBS strain collections (15, 24, 33). However, as many as 9 (16%) of the 56 rib-positive type III strains tested in this study were negative when probed against the R4 specific antibodies, in agreement with the notion that a comparatively large proportion of these strains expressed no or very low levels of the R4 protein. Discrepancy between gene possession and gene expression has been found for GBS R proteins (13), for C~b~ encoded by bac (28), for in vitro mutants containing bca, which encodes C~α~ (29), and for gene clusters which determine capsular polysaccharide synthesis (4, 12). Little is known of the genetic basis of the expression failure except that transcriptional failure was demonstrated in the case of C~b~ expression failure (28) and that phase variation-like genetic mechanisms probably determined the C~α~ expression failure (29). Changes of regulatory genetic elements similar to those found in the C~α~ mutants (29) may determine the rib/R4 discrepancy in the type III GBS found in the present study. One impact of expression failure will be that antibody-based methods for antigen detection will underestimate the prevalence of gene possession by the bacteria. This is important to consider in relation to choice of methods in epidemiological GBS typing.

R4 Mab F39. F39 antibody was produced in our laboratory (1), has been considered R4 specific, and has been used extensively for R4 detection in serotyping of clinical GBS isolates.
and for research purposes (15, 24). To our surprise, the R4 MAb showed a titer of 3,200 in indirect ELISA with both Alp3 and R4 as the coating antigens and showed a positive FAT with both alp3- and rib-positive strains, similar to what occurred with the R4/Alp3 common antibodies, but not with strains which expressed only Ca, Cβ, Alp2, or R3 (not shown). In competition ELISA, the rabbit R4/Alp3 common antibodies blocked the binding of the murine R4 MAb to R4, whereas the R4 specific antibodies had no blocking activity (Fig. 3). When the MAb was tested for its ability to inhibit the binding to R4 of the rabbit R4/Alp3 common antibodies, the signaling in ELISA generated by the polyclonal antibodies was not affected (not shown). These findings are consistent with the notion that the R4 MAb targeted an epitope within the area of the R4/Alp3 common determinant and that this area, as defined by means of the polyclonal antibodies, included other epitopes in addition to the R4 MAb epitope. These results also showed that animals of different species responded immunologically to the same R4 region, the R4/Alp3 common site. If humans also respond to this region is not known. The reason we misjudged the specificity of the R4 MAb (1) was that we used the reference strain ATCC 12403 (III/R1alp2) to exclude R1 protein recognition by the antibody. However, strain 12403 expresses Alp2, which is devoid of the R4 MAb target (19). The existence of Alp3 was unknown at that time. The R4 MAb can no longer be used as an R4-specific reagent, but it still may have important potential, for instance, in studies of the immunobiological function of the R4/Alp3 common determinant and of antibodies against it.

Reference strain NCTC 9828 (Compton; Prague 25/60). Strain 9828 (NT/R3, R4) has been used extensively as a reference strain for the GBS proteins R3 and R4. Also, a “new” R-like protein from this strain was described recently (6). When tested by us in the absorption ELISA, strain 9828 and the R4 prototype strain 65604 showed equally strong capacities to bind the R4/Alp3 common antibodies (Fig. 4, lines c and d). The R4/Alp3 common antibodies, as well as the R4 MAb, were also bound by 9828 in the FAT and in Western blotting, and strain 9828 also formed ladder-like banding patterns with these antibodies, essentially like strain 65604 (Fig. 2B). However, strain 9828, as distinct from strain 65604, showed only weak, if any, binding of the R4 specific antibody in the absorption ELISA (Fig. 4, lines a and b) and was negative against this antibody in the FAT and Western blotting (not shown). Strain 9828 also failed to bind antibodies against the antigenic determinant Alp2/Alp3 common (not shown), a determinant shared by Alp2 and Alp3 (19). Thus, strain 9828 contained the antigenic marker R4/Alp3 common, which can be a part of either Alp3 or R4, but showed negative results with respect to other antigenic markers of these proteins. We found that strain 9828 was negative by alp2, alp3, and rib PCR, as was also found by investigators who proposed that this strain possessed a “new” alp gene called alp4 (13), which might encode the protein targeted by the R4/Alp3 common antibodies. Another possibility could be that strain 9828 originally possessed a normal rib gene which had undergone mutational changes, resulting in a truncated gene, and that this has affected both the region which encoded the antigenic R4 specific site and the primer binding sites. Our findings have substantiated the notion, based on PCR and amplicon sequencing (13), that strain 9828 diverged markedly from features which hitherto have been attributed to it, which should not be the case with a reference strain. Actual expression by 9828 of strain-variable laddering proteins, however, needs further clarification.

Comments. This study has shown that the R4 protein harbors two antigenic determinants, R4 specific and R4/Alp3 common. The possibility that R4 possesses additional antigenic sites cannot be excluded. Both of the defined antigenic determinants may be important in the context of GBS-induced disease, for instance, in the induction of protective antibodies. Antisera raised against purified Rib/R4 have protective activity in animal models (31, 32, 33), but the contribution to this activity of antibodies against each of the R4 determinants has not been fully elucidated. Expression of the R4/Alp3 common site, encoded either by alp3 or by rib, occurs frequently in GBS, notably in pathogenic serotype III, V, and VIII strains (7, 13,
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