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# Equivalence of high-virulence clonotypes of serotype III group B *Streptococcus agalactiae* (GBS)

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Analysis of growth characteristics, multilocus enzyme electrophoresis, restriction digest pattern (RDP) typing and multilocus sequence typing have identified clonotypes of serotype III group B *Streptococcus agalactiae* (GBS) associated with invasive infection in neonates. This study sought to unify phenotypic and genotypic classifications of type III GBS strains associated with increased virulence in newborns. High-virulence clonotype (HVC) strains possessed the translation initiation factor 2 (*infB*) C allele, found in RDP type III-3 strains, and hybridized with the RDP type III-3-specific probe AA3.6, whereas non-HVC strains shared the *infB* A allele and genomic DNA from these strains did not hybridize with the AA3.6 probe. The characteristic growth lag of HVC GBS at 40 °C has been attributed to the presence of a heat-labile fructose-1,6-bisphosphate aldolase (Fba) enzyme in these strains. The deduced amino acid sequence of *fba* genes of both HVC and non-HVC strains, however, were identical. HVC and RDP type III-3 represent the same genetically related group of bacteria. The characteristic growth differences of virulent strains of type III GBS, however, are not directly attributable to differences in *fba*.

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## INTRODUCTION

Group B *Streptococcus agalactiae* (GBS) are classified into nine serotypes, based on the structure of the capsular polysaccharide (Pattison *et al.*, 1955). Type III GBS are of particular interest, since these organisms are responsible for much disease in neonates and their mothers (Baker, 2000).

GBS possess a highly clonal population structure, with each serotype of bacteria composed of one to four genetically related subgroups (Takahashi *et al.*, 2002). Several investigators have identified differences in the pathogenic potential of certain GBS clonotypes (Mattingly *et al.*, 1990; Maurer & Mattingly 1988; Musser *et al.*, 1989; Nagano *et al.*, 1991; Takahashi *et al.*, 1998, 2002). Maurer and Mattingly first noted that type III GBS strains causing invasive infections in neonates could be distinguished from strains colonizing healthy infants by differences in their growth characteristics in chemically defined medium (FMC; Maurer & Mattingly 1988; Musser *et al.*, 1989). Less-virulent strains that were grown in FMC with 65 mM phosphate to stationary phase rapidly initiated growth in FMC with >125 mM phosphate, whereas disease-associated isolates required a prolonged incubation period prior to regrowth. In subsequent studies, these investigators demonstrated that members of the high-virulence clonotype (HVC), in addition to the growth lag in FMC with high phosphate, also grew poorly at 40 °C, whereas less-virulent strains had similar growth rates at 37 and 40 °C (Palacios *et al.*, 1999). The poor growth of HVC strains at elevated temperatures was attributed to instability of the glycolytic enzyme fructose-1,6-bisphosphate aldolase (Fba; Mattingly & Eskew, 1993).

Recently, we have used restriction endonuclease digest patterns (RDPs) and multilocus sequence typing (MLST) to define the population structure of GBS (Takahashi *et al.*, 1998, 2002; Jones *et al.*, 2003). RDP identified two major clonal subgroups of serotype III GBS; types III-2 and III-3 (Takahashi *et al.*, 2002). In both Salt Lake City and Japan, RDP type III-3 GBS caused most invasive neonatal infections. Thus, classifications of GBS by both phenotypic characteristics and more recent genotypic analyses each

Abbreviations: Fba, fructose-1,6-bisphosphate aldolase; GBS, group B *Streptococcus agalactiae*; HVC, high-virulence clonotype; RDP, restriction digest pattern.

The GenBank accession numbers for the *fba* gene sequences described in this study are AY228464–AY228467.

identify a subgroup of type III GBS strains associated with disease in human infants. We sought to unify these findings by directly determining whether these two high virulence subgroups represent the same genetically related strains.

## METHODS

**Bacteria.** GBS 107 and 110 are type III strains that exhibit the poor growth in FMC at 40 °C that is characteristic of HVC strains (Palacios *et al.*, 1999). GBS strains 32R and 181 are non-HVC strains that grow well under these conditions. Bacteria were grown in Todd–Hewitt broth (THB) or on Columbia blood agar plates.

**Analysis of *infB* alleles.** RDP type III-3 GBS each possess the translation initiation factor 2 (*infB*) C allele, whereas other RDP types possess the A allele. The central portion of the *infB* gene was amplified from bacterial DNA and sequenced by PCR using oligonucleotide primers and conditions described by Hedegaard *et al.* (2000).

**Detection of RDP type III-3-specific DNA.** RDP type III-3 probe AA3.6 was identified by subtractive hybridization of genomic DNA between a virulent RDP type III-3 strain and a less-virulent RDP type III-2 isolate (Bohnsack *et al.*, 2002). DNA homologous to this probe is present in RDP III-3 GBS strains but not in less-virulent RDP type III-2 strains; therefore, this probe can be used for rapid genotyping of type III GBS.

Southern dot-blot hybridization to detect RDP type III-3-specific DNA was performed with AA3.6 as described previously (Bohnsack *et al.*, 2002). As a positive control, identical dot blots were hybridized with the full-length gene encoding C5a peptidase, *scpB* (provided by P. Cleary, University of Minnesota), which is ubiquitous in human isolates of GBS (Bohnsack *et al.*, 2000).

**Amplification and sequencing of GBS *fba*.** The coding region of *fba* from GBS strain 110 was amplified from genomic DNA using primers corresponding to the *fba* gene *Streptococcus pneumoniae* strain R6. PCR was performed using the following primers: ALDUP 5′-GTGCTA GAATTAACATGTAAGTGGGC-3′ and ALDDN 5′-CACACAGGA AACAGCTATGACCATG-3′ (Hoskins *et al.*, 2001). The amplification product was cloned into pCR2.1 phagemid (Invitrogen) and sequenced to confirm the correct construct. The complete *fba* insert was excised from the vector by digestion with *Eco*RI and purified for use as an *fba* probe.

To obtain the sequences of the 5′ and 3′ ends of the GBS *fba* coding region, a GBS strain 874391 genomic library was generated by digestion of III-3 strain 874391 genomic DNA with *Eco*RI and ligation into Lambda Zap II phage (Stratagene). The genomic library was screened with radiolabelled *fba* probe using standard methods and hybridizing plaques were purified. Genomic DNA fragments containing 5′ and 3′ portions of the *fba* gene and their flanking regions were excised with ExAssist helper phage (Stratagene) to generate subclones in pBS SK− phagemid vectors for sequencing.

Amplification primers corresponding to 5′ and 3′ flanking regions were designed and the entire *fba* coding sequence was amplified from each of the HVC and non-HVC GBS strains. PCR was performed using the following primers: ALD5 5′-ATGGCAATCGTTTCAGCAGAA-3′ and ALD3 5′-AACTATTATCTGTTTATGTTAATTA-3′. Amplification products were sequenced directly.

## RESULTS AND DISCUSSION

HVC strains 110 and 107 possessed the *infB* C allele (data not shown) found in all RDP type III-3 strains (Takahashi *et al.*, 2002). Non-HVC strains 181 and 32R contain the *infB* A allele characteristic of non-RDP type III-3 strains. RDP type III-3-specific probe AA3.6 hybridized with genomic DNA from HVC clone strains 107 and 110 but did not hybridize with non-HVC strains 32R and 181 (Fig. 1). As expected, the *scpB* probe hybridized with DNA from all four strains.

The nucleic acid sequences of the *fba* genes of HVC strains 110 and 107 and non-HVC strains 181 and 32R were identical to one another (data not shown) and to the recently reported serotype III GBS strain NEM316 and type V strain 2603V/R *fba* genes (Glaser *et al.*, 2002; Tettelin *et al.*, 2002). Thus, no shared nucleotide or amino acid differences distinguished HVC from non-HVC *fba* sequences.

Several different groups have identified subgroups of type III GBS that are particularly likely to cause disease in newborns. Musser *et al.* (1989) examined 63 type III isolates from various geographical locations in the USA, and found that these bacteria expressed a distinct multilocus enzyme electrophoresis type (ET 1), grew poorly in FMC with 200 mM phosphate and were commonly recovered from ill neonates. In contrast, 52 % of bacteria classified into other electrophoresis types did not have growth inhibited by high concentrations of phosphate, and these strains were less likely to be isolated from symptomatic patients. Subsequently, Mattingly *et al.* (1990) demonstrated that the same virulent type III strains that grow poorly in FMC with 200 mM phosphate also grew poorly at 40 °C in FMC with 65 mM phosphate.

We have used genetic approaches to define the population structure of type III GBS. Isolates can be divided by analysis of RDP and MLST into two major subgroups (Nagano *et al.*, 1991; Takahashi *et al.*, 1998, 2002, Jones *et al.*, 2003). Most strains causing invasive neonatal disease belong to RDP type III-3/ST-17. These strains contain genetic material that is not present in less-virulent clonotypes of bacteria and that may contribute to their virulence (Bohnsack *et al.*, 2002). Examination of a small number of RDP type III-3 strains suggested that these bacteria have the unusual growth characteristics shared by previously identified HVC strains – 10/10 isolates

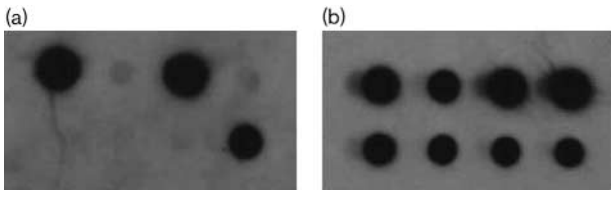


**Fig. 1.** Hybridization of GBS genomic DNA with RDP type III-3-specific probe AA3.6. Five micrograms of genomic DNA from HVC and non-HVC type III GBS strains was denatured and applied to nylon membrane with a 96-well vacuum manifold (top row, left to right: strains 110, 181, 107 and 32R; bottom row, left to right: RDP type III-1 strain 52081, RDP type III-2 strains 53008 and i13 and RDP type III-3 strain 874391). Membranes were hybridized with the RDP type III-3-specific probe AA3.6 (a) or an *scpB* probe (b).

exhibited a lag in growth in FMC with 200 mM phosphate – but the relationship between HVC of type III GBS as defined by phenotypic and genotypic assays has not been formally determined (Nagano *et al.*, 1991). In this study, HVC strains 107 and 110 possessed the *infB* C allele found in virulent RDP type III-3 strains and DNA from these strains hybridized with an RDP type III-3-specific probe, confirming that both phenotypic and genotypic classifications identify the same group of genetically related isolates.

Mattingly & Eskew (1993) found that crude enzyme preparations of Fba from HVC GBS were temperature sensitive, with incubation at 40 °C reducing the aldolase activity of HVC cell extracts by 75 %. Moreover, addition of glyceraldehyde 3-phosphate or 3-phosphoglycerate, products of the aldolase reaction, to heat-treated HVC GBS restored the ability of the bacteria to grow at 40 °C (Mattingly & Eskew, 1993). We found no differences between the *fba* genes of HVC/RDP type III-3 strains and less-virulent type III GBS, suggesting that differences in Fba itself are not responsible for the distinctive growth characteristics of these bacteria. Recent work in *Escherichia coli* suggests that the growth delay in the HVC may result from abnormalities in the assembly of the enzyme at high temperatures. *E. coli* Fba functions as a homodimer and has an absolute requirement for a divalent cation (usually $Zn^{2+}$; Cooper *et al.*, 1996). Heat denaturation and aggregation of this enzyme is a reversible process, facilitated by the molecular chaperones DnaK/DnaJ and enhanced by GroEL/GroES (Kedzierska *et al.*, 2001). Kedzierska *et al.* (2001) hypothesized that denaturation of *E. coli* Fba *in vivo* is caused by a temporary limitation of the DnaK/DnaJ supply, and found that mutations in these systems prevented normal reassembly of thermally inactivated Fba. An analogous system may exist in GBS, with the characteristic poor growth of HVC strains at 40 °C caused by a mutation in chaperones or other proteins involved in reassembling or stabilizing Fba at high temperatures. Alternatively, post-translational modification of Fba by HVC and non-HVC strains may differ, resulting in variation in the enzyme stability.

In summary, we have demonstrated directly that phenotypic and genotypic systems of identifying strains of type III GBS with increased virulence identify the same genetically related group of bacteria. The *fba* genes of these bacteria do not differ significantly from those of less-virulent strains, suggesting that the characteristic poor growth of HVC strains at 40 °C is not directly related to primary Fba structure.

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