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Molecular Characterization of Nonhemolytic and Nonpigmented Group B Streptococci Responsible for Human Invasive Infections

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Group B *Streptococcus* (GBS) is a common commensal bacterium in adults, but is also the leading cause of invasive bacterial infections in neonates in developed countries. The β-hemolysin/cytolysin (β-h/c), which is always associated with the production of an orange-to-red pigment, is a major virulence factor that is also used for GBS diagnosis. A collection of 1,776 independent clinical GBS strains isolated in France between 2006 and 2013 was evaluated on specific medium for β-h/c activity and pigment production. The genomic sequences of nonhemolytic and nonpigmented (NH/NP) strains were analyzed to identify the molecular basis of this phenotype. Gene deletions or complementations were carried out to confirm the genotype-phenotype association. Sixty-three GBS strains (3.5%) were NH/NP, and 47 of these (74.6%) originated from invasive infections, including bacteremia and meningitis, in neonates or adults. The mutations are localized predominantly in the *cyl* operon, encoding the β-h/c pigment biosynthetic pathway and, in the *abx1* gene, encoding a CovSR regulator partner. In conclusion, although usually associated with GBS virulence, β-h/c pigment production is not absolutely required to cause human invasive infections. Caution should therefore be taken in the use of hemolysis and pigmentation as criteria for GBS diagnosis in routine clinical laboratory settings.

Streptococcus agalactiae, or group B *Streptococcus* (GBS), is a Gram-positive bacterium that is usually an asymptomatic member of the human intestinal and vaginal microbiota. However, GBS is also the leading cause of neonatal sepsis and meningitis in developed countries (1–3) and an emerging pathogen in adults, mostly in the elderly (4, 5). Neonatal infections occur predominantly during delivery by inhalation or ingestion of contaminated secretions of the mother's vagina. Rapid invasive infections may follow, with a mortality rate of up to 10% during the first week of life (early-onset disease [EOD], age of 0 to 6 days) or during the first 3 months of life (late-onset disease [LOD], age of 7 to 89 days). Systematic GBS screening prior to delivery and intrapartum antibiotic prophylaxis have efficiently reduced EOD but not LOD (6), highlighting the need to further improve the diagnosis and treatments.

GBS is an extracellular pathogen and one of its characteristics is the secretion of a potent β-hemolysin/cytolysin (β-h/c) (7–9). This β-h/c has a central role in balancing the pro- and anti-inflammatory responses of the infected host and is necessary to breach the epithelial and endothelial barriers and the phagolysosome membrane (10–15). Remarkably, the GBS β-h/c is unique among Gram-positive pathogens and its production is linked to that of a characteristic orange-to-red pigment. This dual and specific β-h/c and pigment phenotype is routinely used in clinical settings to identify GBS isolates (16, 17).

It was initially proposed that the GBS protein CylE was, in fact, β-h/c and that the pigment was a distinct carotenoid-like molecule (18, 19). However, it was recently established that the β-h/c and the pigment correspond to a unique molecule called granadaene (9, 11), a 676-Da ornithine rhamno-polyene (11, 20). Granadaene may be entirely synthesized by the 12 genes of the *cyl* operon (11, 19, 21). It involves the initial synthesis of the β-h/c

core lipid by AcpC, CylD, CylI, and CylG (i.e., the Cyl homologues of fatty acid synthesis enzymes) followed by addition of one ornithine and one rhamnose residue by CylE and CylJ, respectively (11). Finally, export of the β-h/c toxin requires the ABC-type transporter encoded by *cylA* and *cylB* (22).

Transcription of the *cyl* operon is controlled by CovSR (Control of virulence Sensor and Regulator), a two-component system and the major regulator of GBS virulence (23, 24). The transcriptional regulator CovR binds directly to the *cyl* promoter, thereby inhibiting operon transcription (23, 25). CovR activity is itself regulated by phosphorylation by its cognate histidine kinase CovS. In addition, two other proteins control CovR: the serine/threonine kinase Stk1, which also phosphorylates CovR, but not at the same site as that modified by CovS (25, 26), and the transmembrane protein Abx1, which interacts with CovS to control the balance between its kinase and phosphatase activities (27). This regulatory network has been proposed to be the central mediator of
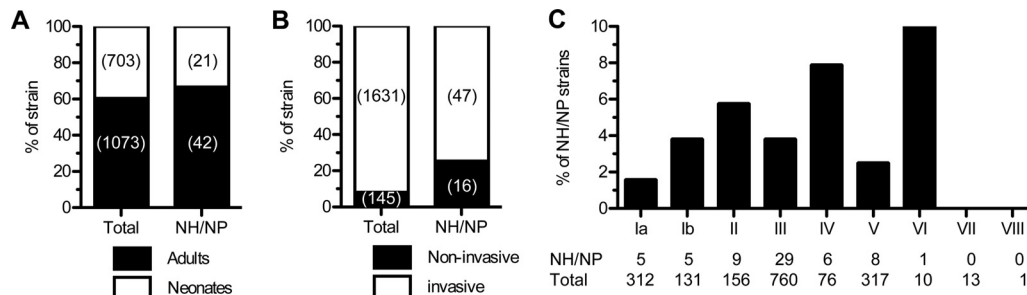
FIG 1 Distribution of NH/NP GBS clinical isolates. Percentages and numbers of clinical GBS strains according to the age of the patient (A), the strain invasiveness (B), and the capsular serotype (C) for the whole collection and for NH/NP strains.

the switch between the commensal and the virulent GBS lifestyles (26, 27).

Independent studies clearly demonstrated that β-h/c pigment production is associated with virulence (9–15). However, it is currently unclear if β-h/c pigment production correlates strictly with GBS virulence. To date, it has been estimated that 85 to 98% of strains isolated from humans produce the β-h/c pigment (16, 28, 29), and it is assumed that nonhemolytic and nonpigmented (NH/NP) strains rarely cause infection (30, 31). Four NH/NP GBS clinical isolates have been characterized in which the *cyl* operon was inactivated by the insertion element IS*1381* (21, 30). In this study, we report the screening of a large collection of human GBS isolates for the presence or absence of the β-h/c pigment. Among 1,776 isolates, 63 (3.5%) were NH/NP. The causative mutations of these NH/NP GBS were identified by whole-genome sequencing or by PCR sequencing of genes known to be involved in β-h/c pigment production. Several as yet uncharacterized genes of the *cyl* operon were also inactivated to accurately characterize and compare the phenotypes of the mutants generated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. GBS clinical strains from noninvasive and invasive infections (positive blood culture, cerebrospinal fluid, or other normally sterile sites) were obtained from the French National Reference Center for Streptococci (www.cnr-strep.fr; Paris, France), and their origins are summarized in Table S1 in the supplemental material. GBS cultures were grown at 37°C in Todd-Hewitt (TH) agar or broth (Difco Laboratories) under static conditions or anaerobically on Granada agar (bioMérieux, France) (16) or Columbia horse blood (5%) agar plates (bioMérieux) When appropriate, erythromycin (10 μg/ml) or kanamycin (1,000 μg/ml) was added to the medium. *Escherichia coli* XL1-Blue (Stratagene) and TOP10 (Invitrogen) were cultured in Luria-Bertani (LB) broth at 37°C with shaking. When appropriate, erythromycin (150 μg/ml) or ticarcillin (100 μg/ml) was added to the medium.

Confirmation of NH/NP GBS strains. The NH/NP phenotypes of GBS strains were confirmed by spotting serial dilutions of an overnight culture on blood and Granada agar plates, as described previously (27). The NH/NP strains were further confirmed as GBS by colony morphology, Lancefield grouping with type B antisera (Oxoid), and matrix-assisted laser desorption ionization−time of flight (MALDI-TOF) mass spectrometry (Bruker) according to the manufacturer's recommendations. Molecular capsular typing was performed by multiplex PCR as described previously (32). Multilocus sequence typing (MLST) was performed *in silico* from whole-genome sequences or by PCR amplification and sequencing as described previously (33); the sequence type (ST) assignment was determined using the reference GBS MLST website (http://pubmlst.org/sagalactiae/).

Genome sequencing. Genomic DNAs were purified (DNeasy blood and tissue kit [Qiagen] or MasterPure Gram-positive DNA purification kit [Epicentre Biotechnologies]) from 10 ml of overnight cultures in TH agar. Whole-genome sequencing was performed at the Sequencing Core Facilities of Institut Pasteur (Paris, France) and at Institut Cochin (Paris, France) using, respectively, Illumina HiSeq and Life Technology Ion Proton systems. Libraries were prepared using an Illumina TrueSeq DNA LT sample prep kit or an Ion Plus fragment kit according to the manufacturers' instructions. We obtained genomic paired-end reads of 2 × 95 nucleotides (HiSeq) or 150-nucleotide reads (Ion Proton). After trimming and quality assessments, short reads were assembled with Geneious software (Biomatters Ltd. [34]) using the NEM316 genome (NCBI NC_004368.1 [35]) as a reference. Contigs encompassing the *cyl*, *abx1*, *covS*, *covR*, and *stk1* locus were extracted and screened for mutations resulting in frameshifts (microdeletions or microinsertions), nonsense (SNPs leading to premature stop codons), or protein length modifications (insertion elements and deletions). Relevant mutations were confirmed by PCR with high-fidelity *Taq* polymerase (Phusion; Thermo Scientific) and sequencing.

DNA manipulations and mutant constructions. The bacterial strains, plasmids, and primers used in this study are listed in Supplemental Tables S2, S3, and S4, respectively, in the supplemental material. GBS mutants were constructed as described previously (27, 36). Briefly, in-frame deletions of *cylX*, *cylD*, *cylG*, *cylAB*, and *cylI* were constructed in the reference strain NEM316 (35). Deletion of the IS*1381* element was constructed in the clinical isolate CCH1084. The expected Δ*cylX*, Δ*cylD*, Δ*cylG*, Δ*cylAB*, Δ*cylI*, and ΔIS*1381* mutants were confirmed by sequencing the relevant loci after PCR amplification.

With use of specific primers (see Table S4 in the supplemental material), the IS*1381* element and flanking chromosomal sequences were amplified by PCR from CCH1084 DNA and cloned into pG1. Integration of the IS*1381* element into the NEM316 chromosome at the same location as in the parental CCH1084 strain was done by homologous recombination as described previously (27, 36).

The pTCVΩP_cyl+_abx1 vector that constitutively expresses a wild-type (WT) *abx1* allele (27) was used to complement the NH/NP strains producing a truncated Abx1 protein. The complementing vector was transferred by conjugation using the *E. coli* HB101/pRK24 donor strain to clinical NH/NP GBS strains as described previously (37), and transconjugants were selected with nalidixic acid (50 μg/ml) for the donor and kanamycin (1000 μg/ml) for the recipient.

RESULTS

Prevalence of NH/NP GBS strains among clinical isolates. A total of 1,776 nonredundant clinical GBS strains isolated in France between 2006 and 2013, from various geographical areas were included in this study. All strains isolated from adults and neonates were screened for β-h/c activity and pigment production. Sixty-three (3.5%) strains, whose prevalence was slightly higher in

TABLE 1 Origins of GBS NH/NP isolates according to their capsular serotype

Origin	Results for capsular serotype[a]:							
	Ia	Ib	II	III	IV	V	VI	Total
From neonates								
EOD meningitis				1 (1)				1 (1)
EOD bacteremia		1 (0)		2 (1)			1 (0)	4 (1)
LOD meningitis				3 (3)				3 (3)
LOD bacteremia	1 (1)			6 (4)	1 (1)			8 (6)
Colonization	3 (0)			1 (0)	1 (0)			5 (0)
Total neonates	4 (1)	1 (0)	0	13 (9)	2 (1)	0	1 (0)	21 (11)
From adults								
Meningitis				1 (1)				1 (1)
Bacteremia		3 (1)	6 (4)	5 (2)	2 (1)	6 (2)		22 (10)
Endocarditis				1 (1)		1 (1)		2 (2)
Bone and joint infection		1 (1)	2 (0)	1 (0)		1 (1)		5 (2)
Others	1 (1)		1 (1)	7 (5)	2 (1)			11 (8)
Total adults	1 (1)	4 (2)	9 (5)	16 (9)	4 (2)	8 (4)	0	42 (23)
Total NH/NP	5 (2)	5 (2)	9 (5)	29 (18)	6 (3)	8 (4)	1 (0)	63 (34)

[a] Data are the numbers of strains for a given capsular serotype and, in parentheses, the numbers of strains selected for sequencing.

adults (42/1,073 = 3.9%) than in neonates (21/703 = 3%) were NH/NP (Fig. 1A). Notably, NH/NP strains were about four times more frequently associated with noninvasive diseases or colonization (16/145 = 11%) than with invasive infections (47/1,631 = 2.9%) (Fig. 1B). The NH/NP strains were isolated from all types of clinical manifestations caused by GBS (Table 1 and Table S1 in the supplemental material). Most strains from adult invasive infections were from bacteremia (22/31 = 71%). Sixteen strains from neonates had elicited invasive infection syndromes ($n = 5$ EOD and 11 LOD) and, among these, 4 ($n = 1$ EOD and 3 LOD) were responsible for meningitis (Table 1 and Table S1 in the supplemental material).

To determine if NH/NP strains comprise a specific subpopulation of GBS, molecular capsule (capsular serotype [CPS]) typing was performed on the whole collection, and the ratio of NH/NP isolates per CPS type was determined (Fig. 1C). For the 6 clinically representative CPS types (Ia, Ib, II, III, IV, and V), all containing at least 76 isolates, the percentage of NH/NP strains ranged between 1.6% (CPS type Ia, 5/312) and 7.9% (CPS type IV, 6/76) (Fig. 1C). Most strains were CPS type III, the major CPS type associated with neonatal invasive infection (1–3), among which 3.8% (29/760) were NH/NP. The remaining 24 strains were CPS type VI ($n = 10$), VII ($n = 13$), and VIII ($n = 1$) and the single NH/NP was CPS type VI. Finally, molecular typing by MLST on a subset of 34 strains representative of the 63 NH/NP clinical isolates revealed several sequence types (ST), including 12 strains belonging to the hypervirulent ST17 lineage responsible for the majority of neonatal meningitis cases (Table 2 and Table S1 in the supplemental material). These analyses show that NH/NP strains are distributed among the major CPS and sequence types responsible for infection.

Association of the NH/NP phenotype with mutations in the _cyl_ operon and _abx1_ gene. To elucidate the molecular basis of the naturally occurring NH/NP phenotype, genome sequences of the 34 representative selected isolates were determined and compared to the genomes of GBS reference strains (Table 2 and Fig. 2A). Until now, NH/NP GBS mutants were constructed by intro-

ducing mutations within the _cyl_ operon (11, 19, 27, 36, 38) or in genes coding its cognate transcriptional regulators, CovSR, Stk1, or Abx1 (27, 38). We therefore focused our analysis on this group of genes (Fig. 2B).

Sequence comparison revealed that 26 (76.5%) NH/NP strains had at least one mutation in the _cyl_ operon, potentially leading to synthesis of truncated proteins (Table 2 and Fig. 2C). These mutations were diverse and include the following: insertion of an insertion element ($n = 8$), deletions or insertions of various lengths ($n = 13$) including 1-bp frameshift insertions or deletions ($n = 6$), and single nucleotide polymorphisms yielding premature stop codons ($n = 5$) (Table 2). Mutations were detected in 9 out of the 12 genes of the _cyl_ operon (Table 2 and Fig. 2C). The most frequently mutated genes were _cylA_, _cylE_, and _cylI_, which were all inactivated in at least 3 strains. Interestingly, all characterized mutations were different, except two IS_1381_ insertions that occurred twice at the same location in _cylA_ (strains CCH1321 and CCH1323) and _cylI_ (strains CCH1344 and CCH1349) genes (Table 2).

The _cyl_ operon of 8 strains was inactivated by insertion sequences IS_1381_ ($n = 6$), IS_Sa4_ ($n = 1$), and ICE_Sp1108_ ($n = 1$). Insertional inactivation of _cylA_ by IS_1381_ was previously described in four clinical isolates (21, 30). Consistently, we identified insertions of IS_1381_ within _cylA_ (3 strains) and additionally in _cylI_ (2 strains). Interestingly, in strain CCH1084 (Table 2), the IS_1381_ insertion occurred between _cylX_ and _cylD_, although neither gene was disrupted. To confirm that this insertion was responsible for the NH/NP phenotype, we demonstrated that restoration of the WT _cyl_ sequence in CCH1084 restored pigmentation and hemolysis, whereas introduction of the same IS_1381_ insertion in NEM316 yielded a very weakly β-h/c mutant strain (Fig. 3A). In strain CCH1339, _cylE_ was inactivated by IS_Sa4_, whereas in strain CCH1327, _cylI_ was disrupted by ICE_Sp1108_. These results uncover numerous different types of events occurring at the _cyl_ locus that are responsible for the NH/NP phenotype in GBS clinical strains.

The analysis of _abx1_, _stk1_, _covS_, and _covR_ sequences revealed

TABLE 2 Loss-of-function mutations in the NH/NP sequenced GBS strains

Isolate	CSP[a]	ST[b]	Mutated gene	Type of mutation	Size or characteristic	DNA position[c]	Protein position[d]
CCH1334	III	17	cylX-D	Deletion		$X1 \to D849$	
CCH1084	III	1	cylX-D	Insertion	IS1381	$X306 \to D1$	
CCH1318	III	17	cylG	Substitution	Stop codon	636/723	213/241
CCH1338	III	366	cylG	Deletion	4 bp	300/723	103/241
CCH1347	III	17	acpC	Substitution	Stop codon	42/306	15/102
CCH1340	IV	459	cylZ	Deletion	1 bp	92/477	55/159
CCH1321	III	106	cylA	Insertion	IS1381	471/930	
CCH1323	III	27	cylA	Insertion	IS1381	471/930	
CCH1337	III	27	cylA	Insertion	IS1381	466/930	
CCH1326	III	17	cylE	Deletion	5 bp	241/2004	81/668
CCH1339	Ia	136	cylE	Insertion	ISSa4	1582/2004	
CCH1341	III	17	cylE	Substitution	Stop codon	1335/2004	446/668
CCH1342	III	27	cylE	Insertion	1 bp	897/2004	304/668
CCH1352	III	17	cylE	Deletion	1 bp	1514/2004	523/668
CCH1345	II	10	cylF	Substitution	Stop codon	595/954	199/318
CCH1333	III	17	cylF-I	Deletion	692 pb	$F749 \to I492$	
CCH1319	III	17	cylI	Deletion	1 bp	1121/2196	386/732
CCH1320	III	17	cylI	Insertion	17 bp	1817/2196	619/732
CCH1322	Ia	23	cylI	Deletion	1 bp	883/2196	306/732
CCH1327	II	28	cylI	Insertion	ICESp1108	1863/2196	
CCH1329	II	12	cylI	Insertion	1 bp	434/2196	158/732
CCH1343	II	22	cylI	Substitution	Stop codon	936/2196	313/732
CCH1344	Ib	8	cylI	Insertion	IS1381	1844/2196	
CCH1349	Ib	1	cylI	Insertion	IS1381	1842/2196	
			abx1	Deletion	1 bp	176/921	73/307
CCH1346	IV	1	cylI	Insertion	12 bp	1172/2196	
			abx1	Substitution	Stop codon	504/921	169/307
CCH1324	V	1	cylI	Deletion	83 bp	1893/2196	
			abx1	Deletion	1 bp	176/921	73/307
CCH1325	III	1	abx1	Deletion	1 bp	176/921	73/307
CCH1330	III	17	abx1	Insertion	1 bp	101/921	53/307
CCH1331	V	1	abx1	Deletion	1 bp	176/921	73/307
CCH1350	V	1	abx1	Deletion	1 bp	176/921	73/307
CCH1328	V	1	abx1	Deletion	1 bp	176/921	73/307
CCH1351	II	2	ND[e]				
CCH1348	IV	459	ND				
CCH1336	III	17	ND				

[a] CSP, capsular serotype.
[b] ST, sequence type by MLST.
[c] Nucleotidic position of the mutation/wild-type gene length.
[d] Amino acid position of the truncation/wild-type protein length.
[e] ND, not determined.

mutations in *abx1* only (Fig. 2 and Table 2). These mutations occurred in 8 strains (23.5%), 4 being of CPS type V (ST-1), which had the same microdeletion, suggesting a mutation hot spot. Three out of the 8 strains had an additional mutation in the *cyl* operon. A plasmid harboring a wild-type copy of *abx1* was introduced in 3 strains in which *abx1* was mutated (CCH1328, CCH1331, and CCH1350). β-h/c pigment production was restored in all three strains (Fig. 3B), confirming that *abx1* mutations were responsible for the NH/NP phenotype.

Overall, of the 34 NH/NP strains analyzed, mutations were present in the *cyl* operon (23/34, 67.6%), in *abx1* (5/34, 14.7%), or in both loci (3/34, 8.8%) (Fig. 2 and Table 2). No mutation that might account for the NH/NP phenotype was found in the 3 (8.8%) remaining strains. This suggests that at least one uncharacterized gene(s) controls β-h/c pigment production in GBS.

**Phenotypic characterization of isogenic *cyl* mutants.** To complete the analysis of the *cyl* operon in the same genetic back-ground, we chose the reference NEM316 strain (35) to generate in-frame deletions of the *cylX*, *cylD*, *cylG*, and *cylI* genes, which encode proteins putatively required for synthesis of the lipid core backbone of the β-h/c pigment (9, 11). We similarly deleted the *cylAB* genes encoding the two subunits of an ABC-like transporter supposedly involved in β-h/c pigment export (22). These mutant strains, as well as previously constructed *cylE*, *cylJ*, and *cylK* NEM316 deletion derivatives for β-h/c pigment maturation (11, 36), enabled us to perform a thorough phenotype-genotype correlation study (Fig. 3C).

Comparison of these isogenic *cyl* mutants revealed various hemolytic and pigmentation phenotypes (Fig. 3C). While the *cylX* mutant was indistinguishable from the parental strain, the *cylE*, *cylG*, *cylAB*, and *cylI* mutants were all nonhemolytic and nonpigmented (Fig. 3C). The *cylD* and *cylK* mutants were faintly pigmented and displayed reduced hemolytic activity. Interestingly, the *cylJ* mutant exhibited slightly reduced pigmentation, contrast-
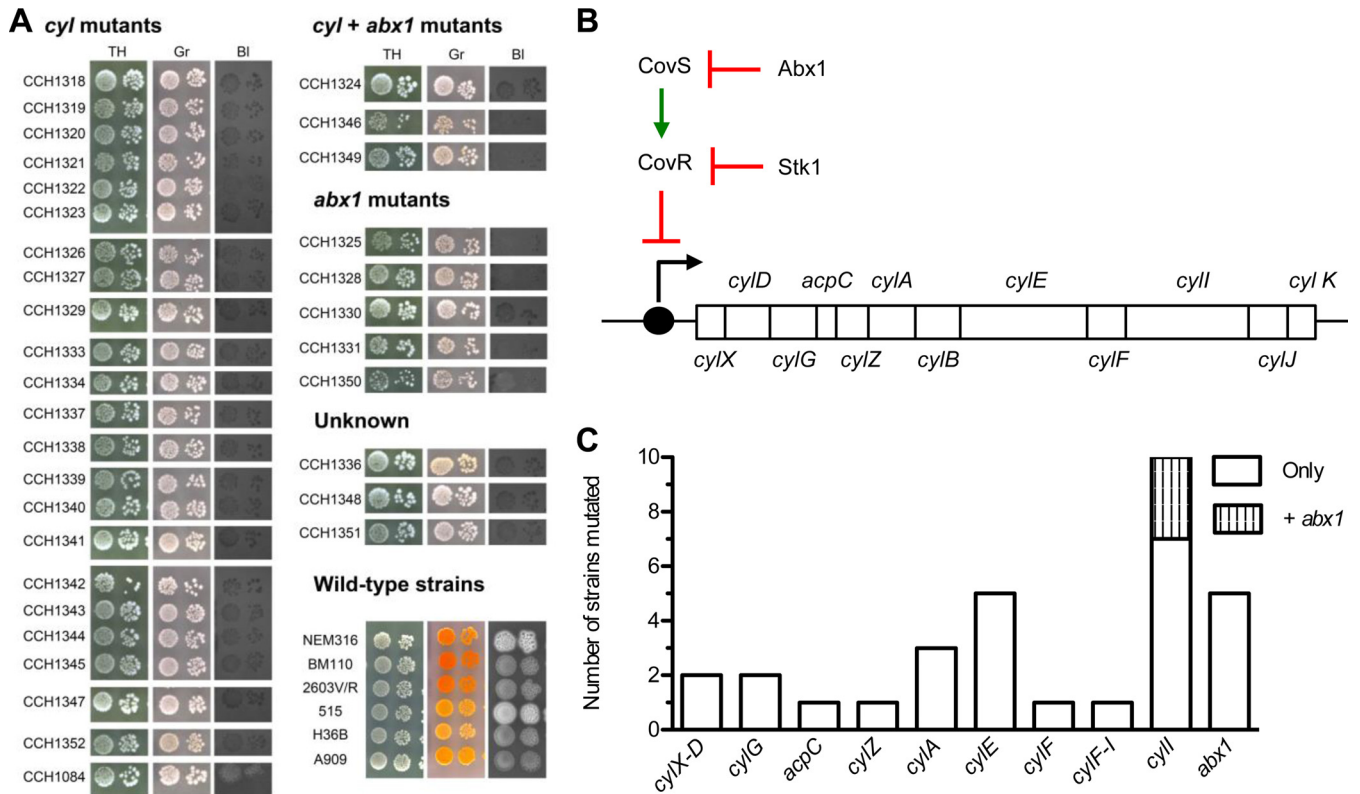
FIG 2 Phenotypes and genotypes of NH/NP clinical isolates. (A) Hemolytic and pigmentation phenotypes of the 34 GBS clinical isolates selected for sequence analysis, organized according to the identified mutation, and of GBS wild-type strains NEM316, BM110, 2603V/R, 515, H36B and A909. TH, Todd-Hewitt agar; Gr, Granada agar; Bl, Columbia agar supplemented with 5% horse blood. (B) Structure and regulation of the *cyl* operon. Red crossed line, transcriptional repression; green straight arrow, activation; black bent arrow, initiation of transcription. (C) Distribution of identified mutations in the *cyl* operon and in the *cyl* regulator gene *abx1* among 31 GBS strains; note that no mutation was associated to the NH/NP phenotype among 3 sequenced strains.

ing with very weak hemolysis (Fig. 3C). Thus, the critical *cyl* genes for β-h/c pigment production in GBS are *cylE*, *cylG*, *cylAB*, and *cylI* and to a lesser extent *cylD* and *cylK*. Mutations in all these genes except *cylK* were found in the sequenced NH/NP GBS clinical isolates (Table 2).
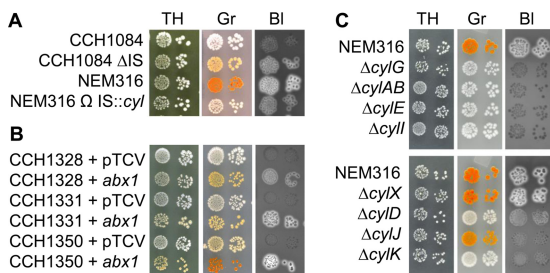
FIG 3 Hemolytic and pigmentation phenotypes in GBS mutants. (A) Effects of deletion of the IS*1381* element from the CCH1084 strain and of the insertion of the same element at the cognate locus in the NEM316 strain. (B) Effects of the genetic complementation of clinical strains mutated only in *abx1* with an empty vector (+ pTCV) and the same vector containing a wild-type copy of *abx1* (+ *abx1*). (C) Pigmentation and hemolysis of the NEM316 reference strain and corresponding in-frame deletion mutants ΔcylG, ΔcylAB, ΔcylE, ΔcylI, ΔcylX, ΔcylD, ΔcylJ, and ΔcylK. Bacterial growth, hemolysis, and pigmentation were observed on Todd-Hewitt agar (TH), Granada agar (Gr), and blood agar (Bl), respectively.

DISCUSSION

The β-hemolytic/cytotoxic activity and the orange-to-red pigmentation are the hallmarks of group B *Streptococcus*. These two phenotypic characteristics have been used historically for GBS diagnosis, and the current view is that they are essential for virulence (8, 9). We tested this assumption by analyzing a large collection of documented GBS human clinical isolates. Sixty-three of the 1,776 isolates were NH/NP, a proportion in agreement with those in previous smaller scale studies (16, 28, 29). Although it is currently thought that NH/NP strains rarely cause infection in humans (30, 31), we observed that NH/NP isolates were found in the most prevalent GBS capsular serotypes and sequence types, including the neonatal hypervirulent ST17 clone (39–41). Remarkably, 47 of the 63 NH/NP strains originated from invasive diseases, including bacteremia and meningitis. These results suggest that β-h/c pigment production is not critical for GBS virulence although numerous *in vitro* and *in vivo* studies using cellular or animal models indicated that greater β-h/c pigment production is associated with increased virulence (10–15, 18).

The pathogenicity of GBS is multifactorial and combines the expression of several factors to result in an overall virulence phenotype that may vary for each isolate (42). In addition, host factors, at the genetic or microbiota level, might contribute to virulence of NH/NP strains that do not express one of the most potent GBS virulence-associated factors. The necessity, but dispensabil-

ity, of β-h/c pigment production for GBS virulence is also illustrated by the greater proportion of NH/NP strains isolated from noninvasive clinical cases (11%) than from invasive disease cases (2.9%). This may suggest that β-h/c pigment production is important for eliciting invasion, but dispensable for the establishment of colonization, in agreement with previous studies on GBS urinary tract infections in animal models (43, 44).

At the genetic level, we identified independent mutations responsible for the NH/NP phenotype, in agreement with the fact that NH/NP strains are independent and not associated with a specific GBS subpopulation. Three-quarters (26/34) of the NH/NP strains harbored a mutation in the *cyl* operon, confirming its central function for the synthesis, maturation, and export of the β-h/c pigment (9, 11, 22, 36). Eight of those strains carry an IS element in the *cyl* operon, among which six correspond to an IS1381 element previously identified in four clinical NH/NP isolates (21, 30). The different locations and nature of the insertion and the different sequence types of the two pairs of strains with an insertion at the same position (in *cylA* genes for CCH1321 and CCH1323 and in *cylI* genes for CCH1344 and CCH1349) suggest that these mutations were acquired independently and that the IS1381 insertion is a fairly common event. The remaining 20 strains have independent mutations in at least 1 of the 12 *cyl* operon genes. While *cylE* inactivation has been shown to systematically yield NH/NP mutants in several unrelated isolates, the phenotype resulting from the inactivation of the other *cyl* genes had not been systematically studied (11, 18, 19, 21, 36). Seven of the 12 genes of the *cyl* operon are thought to be involved in the synthesis of the lipid backbone of the carotenoid-like molecule granadaene (*cylX*, *cylD*, *cylG*, *acpC*, *cylZ*, *cylI*, and *cylK*) (11, 18, 19, 21, 36). While inactivation of *cylG* and *cylI* results in a complete loss of hemolysis and pigmentation, as previously observed with a *cylE* mutant (11, 19, 27, 36), the *cylD* and *cylK* mutants displayed a faint pigmentation and reduced hemolytic activity. CylX, which is involved in the synthesis of malonyl-CoA, is dispensable for hemolysis and pigmentation. These results suggest that CylG and CylI are essential for synthesis of the β-h/c pigment lipid backbone, whereas functional redundancy might exist between CylX, CylD, or CylK and enzymes from other metabolic pathways, in particular the type II fatty acid biosynthetic pathway. The lipid backbone is conjugated to ornithine by CylE and glycosylated by addition of a rhamnose by CylJ. Surprisingly, while *cylE* inactivation yields NH/NP mutants, a *cylJ* mutant exhibits a slightly reduced pigmentation but very weak hemolysis (Fig. 3C). Based on the proposed synthesis pathway (9, 11), this might suggest that the presence of ornithine is essential for both pigmentation and hemolytic activity, whereas the absence of rhamnose mostly affects hemolysis. Further chemical analysis would greatly improve our understanding of the structure-function of this rhamnolipid toxin (9, 11, 20), which is unrelated to the known Gram-positive pore-forming toxins (45–48). Lastly, we confirmed that the ABC transporter encoded by *cylAB* is critical for hemolysis and pigmentation (22).

Mutations relevant to NH/NP isolates were also identified in *abx1*, whose product interacts with the histidine kinase CovS to control activity of the major regulator CovR and, thus, *cyl* transcription (27). While Abx1 mutations have been shown to decrease virulence in animals (27), the characterization of clinical NH/NP strains demonstrates that Abx1 is not absolutely required to cause infections in humans. This suggests that one of the other

regulators (CovS or Stk1) may still respond to environmental signals to activate virulence gene expression in specific tissues. Characterization of these signals is necessary to decipher the regulatory network controlling host-GBS interactions.

In summary, we report the identification of several independent NH/NP GBS strains that retain the capacity to cause invasive infections in humans, including meningitis in the neonate. These strains lost one of their main virulence-associated characteristics, the specific GBS β-h/c pigment molecule, due to mutations in the *cyl* operon or in a *cyl* transcriptional regulator. Thus, while overproduction of the GBS β-h/c pigment is strongly associated with increased GBS virulence, as supported by independent studies, its absence is not synonymous with a loss of virulence, as reported in this study. A direct consequence of this result is the necessity for professionals to be cautious when using pigmentation and hemolysis as the main criteria for GBS diagnosis (17, 29).

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