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Abstract

Group B streptococcus [(GBS or *Streptococcus agalactiae*)] is a leading cause of neonatal meningitis and septicaemia. Most clinical isolates express simultaneously a β-haemolysin/cytolysin and a red polyenic pigment, two phenotypic traits important for GBS identification in medical microbiology. The genetic determinants encoding the GBS haemolysin and pigment have been elucidated and the molecular structure of the pigment has been determined. The *cyl* operon involved in haemolysin and pigment production is regulated by the major two-component system CovS/R, which coordinates the expression of multiple virulence factors of GBS. Genetic analyses indicated strongly that the haemolysin activity was due to a cytolytic toxin encoded by *cylE*. However, the biochemical nature of the GBS haemolysin has remained elusive for almost a century because of its instability during purification procedures. Recently, it has been suggested that the haemolytic and cytolytic activity of GBS is due to the ornithine rhamnopolyenic pigment and not to the CylE protein. Here we review and summarize our current knowledge of the genetics, regulation and biochemistry of these twin GBS phenotypic traits, including their functions as GBS virulence factors.

Introduction

Streptococcus agalactiae [Group B streptococcus, (GBS)] is a leading bacterial agent in neonatal infections, an emerging cause of life-threatening infections in adults and an important pathogen in veterinary medicine. In addition to its role as a pathogen, GBS asymptomatically colonizes the lower gastrointestinal and genitourinary tract of up to 30% of healthy human adults (Ewards & Baker, 2010). In the early 1930s, GBS was first recognized as an animal pathogen and the main cause of bovine mastitis (Sherman, 1937; Keefe, 1997). However, despite its isolation from a number of human sources, including the birth canal (Lancefield, 1933; Lancefield & Hare, 1935), GBS was only reported as a human pathogen by Fry (1938) with the description of three fatal cases of puerperal sepsis. Since the early 1960s (Hood *et al.*, 1961) GBS has been considered a leading cause of neonatal infections, associated with sepsis, meningitis and pneumonia (Verani *et al.*, 2010; Edwards & Nizet, 2011; Rodriguez-Granger *et al.*, 2012). GBS emerged recently as a significant pathogen responsible for invasive infections in adults with predisposing underlying diseases such as diabetes and cancer (Farley, 2001; Ewards & Baker, 2010).

In addition to human and bovine infections, GBS has been isolated from animals such as chickens, camels, dogs, dolphins, horse, lizards, cats, fish, frogs, hamsters, mice and monkeys (Garcia *et al.*, 2008). Thus, in contrast to other streptococcal species that display a fairly restricted host spectrum, GBS can cause infections in a wide range of cold- and warm-blooded animals (Kornblatt *et al.*, 1983; Messier *et al.*, 1995; Evans *et al.*, 2008; Delannoy *et al.*, 2012, 2013; Shuster *et al.*, 2013). Although interspecies transmission of GBS strains among animals has not been demonstrated (Garcia *et al.*, 2008), transmission between human and animal species has been suggested recently (Delannoy *et al.*, 2013).

Streptococcal haemolysins

β-Haemolysins are potent exotoxins that play a key role in the virulence of pyogenic streptococci, such as *Streptococcus pyogenes* [Group A streptococcus, (GAS)], GBS and *S. dysgalactiae* spp. *equisimilis*. One of the most noticeable

phenotypic characteristics of these species is a zone of β-haemolysis surrounding the colonies when grown on blood agar plates (Ayers & Rupp, 1922). Therefore, β-haemolysis as a phenotypic trait has been widely used for the preliminary identification of pyogenic streptococcal species in the clinical laboratory (Facklam *et al.*, 1979; Facklam, 2002; Spellerberg & Brandt, 2011). Despite the similar phenotypic appearance of β-haemolysis in most streptococci from the pyogenic group, the molecular details of these β-haemolysins differ considerably.

GAS produce two different haemolytic toxins, streptolysin S (SLS) and streptolysin O (SLO) (Todd, 1938). SLS is a 2.7-kDa bacteriocin-like peptide, oxygen-stable and nonimmunogenic, encoded by the *sag* gene cluster (Nizet *et al.*, 2000) and responsible for β-haemolysis (Todd, 1938). Its cellular substrates include erythrocytes, leukocytes, platelets and subcellular organelles (Datta *et al.*, 2005; Molloy *et al.*, 2011). SLS-encoding gene clusters are not only present in many streptococcal species (Fuller *et al.*, 2002; Humar *et al.*, 2002; Rato *et al.*, 2011), but have also been found in *Staphylococcus aureus*, *Clostridium botulinum* and *Listeria monocytogenes* (Cotter *et al.*, 2008; Gonzalez *et al.*, 2010) indicating that this toxin is widespread and conserved among gram-positive pathogens. SLO is a 57-kDa protein belonging to the group of thiol-activated cytolysins including listeriolysin O of *L. monocytogenes*, and perfringolysin O (PFO) of *Clostridium perfringens*. Based on the high overall degree of similarity in the primary structure, all members of the family are thought to share a common mechanism of action that involves binding to cholesterol-containing membranes (Billington *et al.*, 2000) followed by insertion, oligomerization of 20–80 monomers, and formation of a pore of 20–30 nm diameter (Dramsi & Cossart, 2002). In contrast to SLS, SLO is an immunogenic protein, and antibodies against SLO are useful for documenting recent exposure to GAS (McCormick *et al.*, 2006) or *S. dysgalactiae* spp. *equisimilis* (Jansen *et al.*, 1999; Brandt & Spellerberg, 2009).

GBS exhibit two different cytolytic toxins, the β-haemolysin and the CAMP (Christie Atkins Munch-Petersen) factor. The CAMP factor is a heat-stable 226-aa protein which is independent of β-haemolysin and pigment production (Marchlewicz & Duncan, 1980; Tapsall & Phillips, 1987). The CAMP factor is not haemolytic *per se*, although it can lyse sheep erythrocytes pretreated with staphylococcal sphingomyelinase. It is used as a diagnostic tool in identifying GBS strains (Christie *et al.*, 1944; Rühlmann *et al.*, 1988; Hensler *et al.*, 2008b). The GBS β-haemolysin accounts for the haemolytic phenotype on blood agar plates. Nevertheless, in spite of the wealth of literature referring to its role in virulence and its mechanism of action (Rajagopal, 2009), the biochemical nature

of the GBS haemolysin has remained elusive until the publication of a recent report indicating that the haemolysin is not a pore-forming toxin but a rhamnolipid identical to the GBS pigment (Whidbey *et al.*, 2013).

GBS β-haemolysin/cytolysin (β-h/c)

Most human GBS strains produce a surface-associated β-h/c, which plays a key role in GBS pathogenesis. It can target a wide spectrum of cells, and hyperproduction of this haemolysin is associated with fulminant disease in clinical GBS cases as well as severe cases of infection in animal models.

Biological characteristics

The prototypical phenotype of GBS clinical isolates displays a narrow zone of β-haemolysis on blood agar plates (Rotta, 1986). GBS haemolysin is primarily a broad-spectrum cytolysin capable of destroying many eukaryotic cells (Tapsall & Phillips, 1991; Nizet *et al.*, 1996). It is therefore referred to as the GBS β-h/c (Doran *et al.*, 2002). In contrast to other well-characterized streptococcal haemolysins (Nizet, 2002), such as SLS and SLO, not much is known about the molecular details responsible for the membrane alterations (Rajagopal, 2009). However, membrane defects observed as a result of exposure of erythrocytes with a haemolytic GBS wild-type strain appear irregular in shape and exhibit different sizes (Fig. 1), suggesting a mechanism different from a classical pore-forming toxin. The cytolytic activity of the GBS β-h/c was shown to be inhibited by phospholipids such as phosphatidylcholine and phosphatidylethanolamine (Marchlewicz & Duncan, 1980; Ferrieri, 1982; Dal & Monteil, 1983; Tapsall & Phillips, 1991; Fettucciari *et al.*, 2011). This led to the hypothesis that GBS β-h/c could have a similar affinity for phospholipids in the eukaryotic cell membrane guiding the toxin to its site of action (Liu & Nizet, 2006).

It has long been assumed that the GBS β-h/c could be a surface-associated protein requiring a direct contact with the target membrane to induce cell lysis (Marchlewicz & Duncan, 1980, 1981; Dal & Monteil, 1983; Platt, 1995; Nizet, 2002; Liu & Nizet, 2004). Haemolytic activity can be extracted from the bacterial surface using molecules such as starch, Tween or bovine serum albumin that act as stabilizer or carrier molecules (Marchlewicz & Duncan, 1980, 1981; Ferrieri, 1982Dal & Monteil, 1983). As the elution profiles of the carrier molecules in gel size-exclusion chromatography did not change in the presence of the haemolysin (Marchlewicz & Duncan, 1980; Tsaihong & Wennerstrom, 1983), it was assumed that GBS haemolysin is a small molecule. As attempts to produce
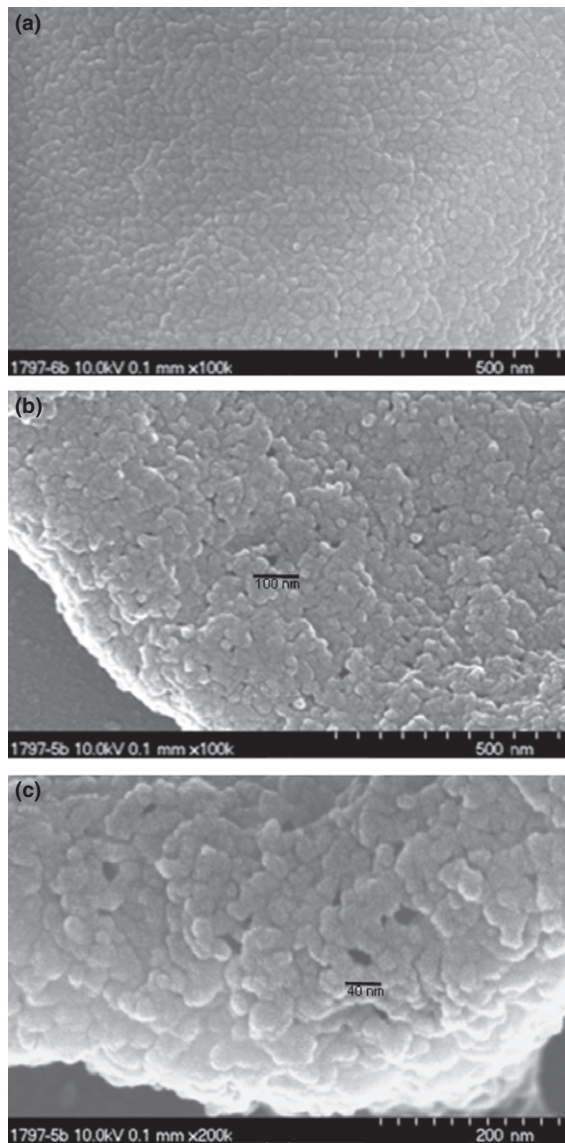
Fig. 1. Electron micrographs of human erythrocytes incubated with haemolytic extracts of the β-haemolytic GBS wild-type strain AC450 (b, c) and a nonhaemolytic GBS mutant (a) carrying an IS*S1* insertion in the *acpC* gene of the *cyl* gene cluster. Preparation of the haemolytic extracts and the generation of the nonhaemolytic mutant strain have been described previously (Spellerberg *et al.*, 1999). A 4% solution of human erythrocytes was incubated with the respective haemolysin extract for 5 min at 17 °C to allow attachment of haemolysin to the erythrocyte membrane, at a temperature at which no haemolysis occurs. Following fixation of the erythrocytes the assay was incubated for 3 min at 37 °C to induce erythrocyte lysis. Images were taken with an Hitachi S 5200 scanning electron microscope at magnifications as indicated.

specific antisera in rabbits with a haemolytic preparation obtained after gel exclusion chromatography of crude bacterial surface extracts had been unsuccessful (Dal &

Monteil, 1983), the GBS haemolysin was characterized as being a nonimmunogenic substance.

Many attempts to purify and study this elusive cytolysin have failed, raising doubts about its proteinaceous nature (Marchlewicz & Duncan, 1980, 1981; Dal & Monteil, 1983; Tsaihong & Wennerstrom, 1983; Nizet *et al.*, 1997a;). The main obstacles encountered in β-haemolysin purification include rapid loss of activity when stored at room temperature (Tapsall & Phillips, 1991), due to its high thermolability (Marchlewicz & Duncan, 1981; Dal & Monteil, 1983), and the loss of haemolytic activity upon detachment from the carrier molecule (Liu & Nizet, 2004). As most studies have been carried out using complex haemolysin-carriers, an accurate analysis has been hampered, leading to the production of confusing results about the proteinaceous nature of the molecule. The biological characteristics of the GBS haemolysin can be summarized as a broad-range surface-associated nonimmunogenic cytolysin of small molecular size displaying a rapid loss of activity.

The *cyl* gene cluster

The genes responsible for β-haemolysis of GBS are encoded in the *cyl* gene cluster Spellerberg *et al.*, 1999, 2000a, b; (Pritzlaff *et al.*, 2001). They were identified by screening transposon mutant libraries of a serotype Ia and a serotype III GBS strain (Spellerberg *et al.*, 1999). Nonhaemolytic mutants were shown to harbour various mutations in a cluster of genes, designated as *cyl* genes in reference to the cytolytic function of the toxin. The link between these genes and β-haemolysin production was substantiated by analysing naturally occurring nonhaemolytic GBS strains. 1–5% of human GBS isolates are nonhaemolytic (Merrit & Jacobs, 1976; Noble *et al.*, 1983; Reardon *et al.*, 1984; Brimil *et al.*, 2006; Adler *et al.*, 2008; Verani *et al.*, 2010) and often harbour insertion sequences (ISs) in one of the *cyl* genes (Spellerberg *et al.*, 1999, 2000b; Sigge *et al.*, 2008). The *cyl* operon, which is made up of 12 genes (*cylX*, *cylD*, *cylG*, *acpC*, *cylZ*, *cylA*, *cylB*, *cylE*, *cylF*, *cylI*, *cylJ*, *cylK*) (Fig. 2), is unique to GBS. The *cyl* operon was initially linked to haemolytic activity (Spellerberg *et al.*, 1999) and later to pigment production by genetic studies of nonpigmented mutants (Spellerberg *et al.*, 2000b). CylD, CylG, ApcC, CylZ (Spellerberg *et al.*, 1999) and CylI (Spellerberg *et al.*, 2000a; Pritzlaff *et al.*, 2001) display homologies with enzymes of prokaryotic fatty acid biosynthesis: CylD with a malonyl-CoA-ACP transacylase, CylG with a 3-ketoacyl-ACP-reductase, ApcC with an acyl carrier protein and CylZ with FabZ enzymes (fatty acid biosynthesis, 3R-hydroxymyristoyl ACP dehydratase). The genes *cylA* and *cylB* encode an ABC (ATP-binding cassette) transporter (Spellerberg *et al.*, 1999).
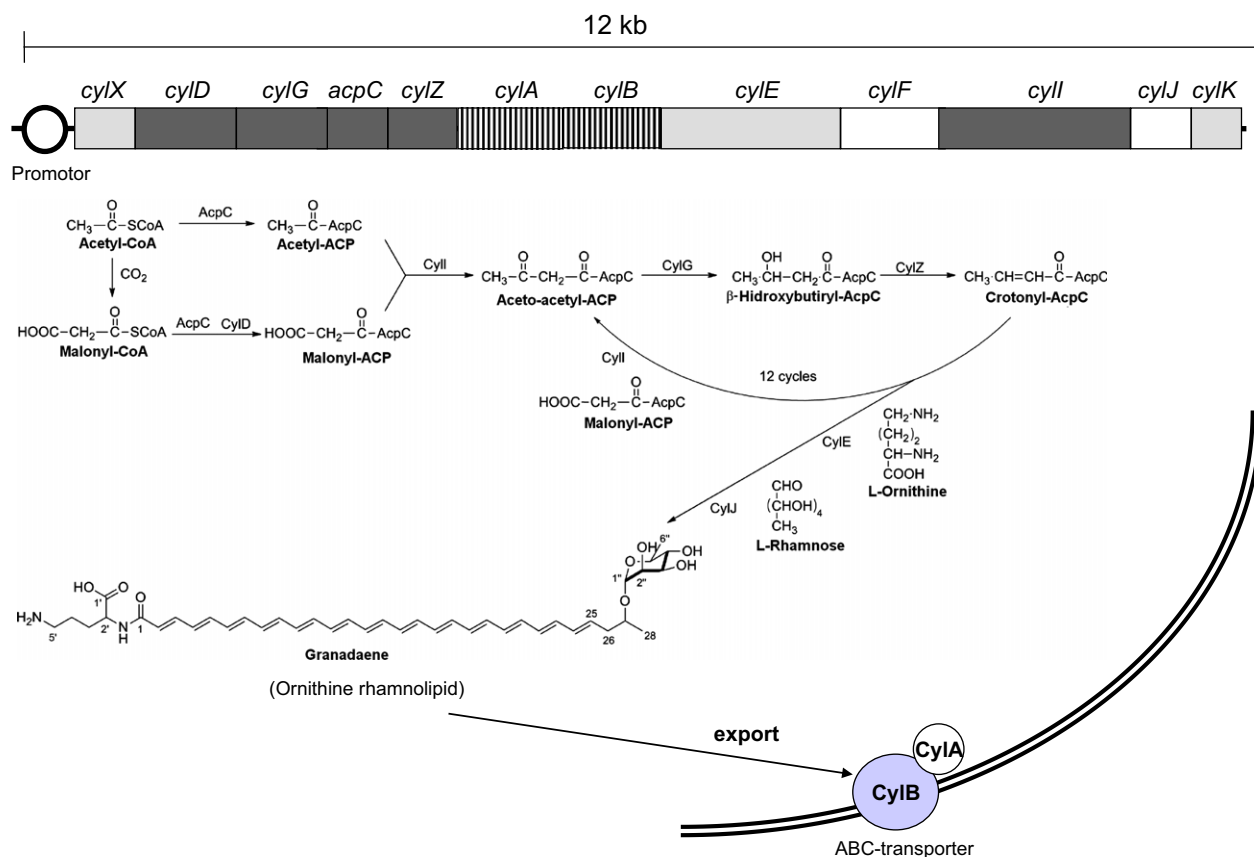
Fig. 2. A representation of the 12 genes belonging to the *cyl* gene cluster of GBS and the theoretical biosynthetic steps toward granadaene formation. The biosynthesis of granadaene should take place by sequential condensation of malonyl-ACP blocs in an iterated cycle of condensation reduction and dehydratation similar to the fatty acid biosynthesis pathway. The *cyl* operon genes coding for the respective enzymes are shown.

The CylAB transporter displays significant similarities to multidrug resistance (MDR) transporters and can export MDR substrates (Gottschalk *et al.*, 2006): *cylX* has been predicted to encode an acetyl coenzyme A (CoA) carboxylase; the *cylE* gene product displays homologies with an *N*-acetyltransferase; *cylF* encodes a putative aminomethyltransferase; CylI displays homologies with a 3-ketoacyl-ACP synthase; CylJ displays homologies with with a glycosyltransferase; and *cylK* encodes a putative phosphopantetheinyl transferase (Spellerberg *et al.*, 1999, 2000a; Pritzlaff *et al.*, 2001; Whidbey *et al.*, 2013). While initial studies found that mutations in different *cyl* genes led to a loss of haemolytic activity (Spellerberg *et al.*, 1999), a subsequent publication demonstrated that only mutation of *cylE* invariably resulted in a nonhaemolytic phenotype that could be restored upon complementation (Pritzlaff *et al.*, 2001). In addition, overexpression of *cylE* in *Escherichia coli* conferred to the recombinant bacteria the ability to lyse erythrocytes. These results suggested strongly that CylE represented the GBS haemolysin. Attempts to purify

and characterize the *cylE* product, a protein of 78.3 kDa, were unsuccessful, and CylE did not show significant homology to any known pore-forming toxin (Pritzlaff *et al.*, 2001).

Moreover, the reintroduction of *cylE* including the adjacent *cylA/B* in a GBS nonhaemolytic mutant harbouring a deletion of the *cyl* cluster did not lead to a restoration of the haemolytic phenotype (Whidbey *et al.*, 2013). Today, sequence analysis supports that CylE is an acyl CoA acyltransferase involved in the biosynthesis of the rhamnolipid (Tettelin *et al.*, 2005; Whidbey *et al.*, 2013; http://www.uniprot.org/uniprot/Q3K232-). Furthermore, attempts to confirm the previous report that recombinant expression of *cylE* in *E. coli* results in a haemolytic phenotype have failed (Whidbey *et al.*, 2013). These findings show that *cylE* is necessary but not sufficient for expression of the haemolysin (Gottschalk *et al.*, 2006; Whidbey *et al.*, 2013).

A recent study challenged the idea of CylE as a pore-forming toxin (Whidbey *et al.*, 2013). This result is supported by the fact that no protein could be found in

haemolytic cell-free extracts of GBS; extracts were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Whidbey *et al.*, 2013) as well as nuclear magnetic resonance (NMR) spectroscopy (M. Rosa-Fraile, unpublished results). The same study demonstrated also that the haemolytic and cytolytic activity of GBS is due to the ornithine rhamnolipid pigment. Interestingly, analogues of the *cyl* operon genes, which is absent in other streptococcal genomes, can be found in bacteria such as *Bacillus* spp., *Actinomyces viscosus* and *Propionibacterium* spp. (Whidbey *et al.*, 2013).

Regulation of the *cyl* operon

The dual nature of GBS – with its ability to shift from a harmless commensal microorganism to a life-threatening pathogen – requires the appropriate regulation of virulence factors in response to different environmental conditions encountered in the host. Regulation of virulence factor expression in bacteria is primarily accomplished by two-component regulatory systems (TCSs) that allow bacteria to adapt to changing environmental conditions (Stock *et al.*, 2000).

A typical TCS consists of a membrane-associated histidine kinase (HK) – with an extracellular input sensor domain and a corresponding cytoplasmic effector domain – and a cytoplasmic response regulator (RR). Specific environmental stimuli provoke a conformational change in the input domain of the HK that causes the activation of its cytoplasmic domain, which autophosphorylates at a specific histidine residue. The phosphate group is then transferred to a specific aspartate residue in the cognate RR. Phosphorylation of the RR controls its activity as a transcriptional activator or repressor of multiple genes and initiates the corresponding cellular responses.

Transcription of the *cyl* operon is tightly controlled by the TCS, CovS/R (control of virulence) also known as CsrR/S (Csr capsule synthesis regulator). Apart from regulating the expression of the *cyl* gene cluster, CovS/R controls several other virulence factors (Lamy *et al.*, 2004; Jiang *et al.*, 2005, 2008; Lembo *et al.*, 2010; Cumley *et al.*, 2012; Patras *et al.*, 2013). CovS phosphorylates the regulator CovR at a conserved aspartate residue (Asp53), which allows binding of CovR to a conserved DNA motif in the *cyl* promoter region repressing *cyl* expression. Therefore, inactivation of the regulator CovR leads to constitutive overexpression of the *cyl* operon, resulting in a hyperhaemolytic and hyperpigmented mutant (Lamy *et al.*, 2004; Jiang *et al.*, 2005). These hyperhaemolytic GBS strains have been linked to fulminant GBS infections in humans (Sendi *et al.*, 2009; Whidbey *et al.*, 2013).

Additional regulatory elements were shown to allow the fine-tuning of CovS/R and therefore indirectly control β-haemolysin expression. GBS encodes a single eukaryotic-type membrane-associated serine/threonine kinase Stk1 and its cognate, soluble protein, serine/threonine phosphatase Stp1 (Rajagopal *et al.*, 2003; Burnside *et al.*, 2011). Stp1 phosphorylates CovR on threonine 65, which decreases the phosphorylation of CovR at Asp53 relieving the CovR-mediated repression of the *cyl* operon. Stk1 positively regulates transcription of β-h/c, which is critical for GBS virulence, and Stk1 mutants produce less β-h/c compared with wild-type strains (Rajagopal *et al.*, 2006; Lin *et al.*, 2009). Stp1 is the cognate phosphatase of Stk1, and indeed Stp1 mutants exhibit several phenotypes such as decreased haemolytic activity, increased autolysis and a reduction in the ability to cause systemic infections (Burnside *et al.*, 2011). Abx1 was recently identified as the third partner of the CovS/R system in GBS through direct interaction with CovS (Firon *et al.*, 2013). RovS, a stand-alone transcriptional regulator, activates the expression of *cylE* and other genes of the *cyl* operon through direct binding to the promoter region (Samen *et al.*, 2006). Thus, multiple signals sensed through CovS, Stk1 and Abx1 are integrated via CovS/R to fine-tune haemolysin expression (Firon *et al.*, 2013; Fig. 3).

GBS pigment

Approximately 95% of all human GBS isolates produce a characteristic brick-red pigment that is unique among streptococci. Expression of the pigment is always linked to the expression of a key virulence factor, the GBS β-haemolysin encoded by a single genetic locus known as the *cyl* operon.

Biological characteristics

The production of a orange to brick-red pigment by human GBS strains is a characteristic phenotypic feature reported very early in the literature (Durand & Giraud, 1923; Sherman, 1937; Plummer, 1941). Fallon (1974) proposed the use of pigment detection as a diagnostic tool for GBS identification. This orange, brick or red pigment is unique and highly specific for GBS isolates and is used in the clinical laboratory for the identification of GBS (Fallon, 1974; Merrit & Jacobs, 1976; Merrit *et al.*, 1976; Noble *et al.*, 1983; Rosa-Fraile *et al.*, 1999b; Spellerberg & Brandt, 2011). Nevertheless, pigment production can be variable among bovine strains and other animal species isolates (Mhalu, 1976; Merritt & Jacobs, 1978; Brglez, 1983; Lämmler *et al.*, 1985; Garcia *et al.*, 2008).

In early studies (Merritt & Jacobs, 1978), GBS pigment was shown to exhibit a three-peak UV–visible absorption
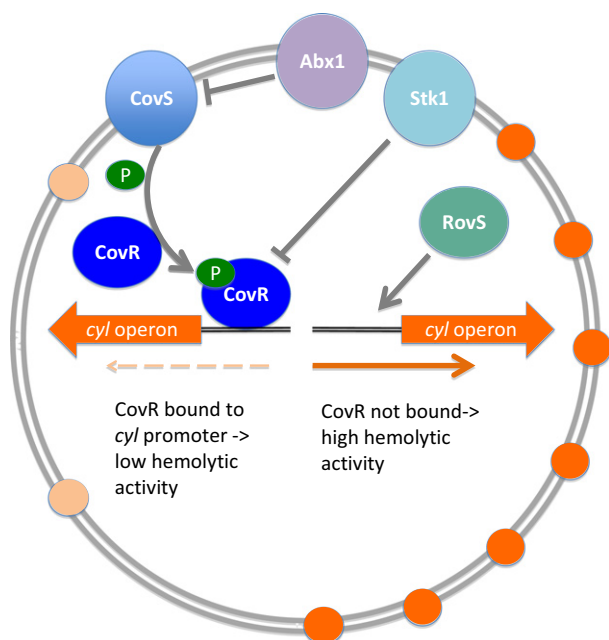
Fig. 3. Regulators controlling transcription of the *cyl* operon. The two-component system CovS/R represents the major regulator of haemolysis and pigmentation in GBS. In wild-type strains the response regulator CovR is phosphorylated through CovS and bound to the *cyl* promotor region. Binding of CovR results in a repression of *cyl* gene transcription. This repression is modulated by an inhibition of CovR through the serine threonine kinase Skt1 and an inhibition of CovS through the Abi domain protein Abx1. In addition, the RovS regulator as a stand-alone system can increase haemolysin and pigment expression through binding to the *cyl* promotor region.

spectrum at 525, 485 and 455 nm strongly resembling the characteristic spectrum of a carotene with 12 double conjugated bonds (Britton, 1995) and thus suggesting for the first time that the GBS pigment is a carotene (Merritt & Jacobs, 1978). Unlike isoprenoid carotenes, which are lipophilic substances (Schiedt & Liaaen-Jensen, 1995), the GBS pigment cannot be extracted from GBS cultures with organic solvents (Merritt & Jacobs, 1978). In addition, the GBS genome sequences (Glaser *et al.*, 2002; Tettelin *et al.*, 2002, 2005) do not contain homologues of phytoene synthases, phytoene or carotene dehydrogenases and lycopene cyclases, which are common enzymes of the carotene biosynthesis pathway (Sieiro *et al.*, 2003). The characterization of GBS pigment structure was hampered for many years by its strong association to the cell wall (Merritt & Jacobs, 1978). It cannot be extracted using water, physiological saline, HCl, alcoholic KOH, methanol, ethanol, acetone, diethyl ether or petroleum ether (Haug & Soderlund, 1977; Merritt & Jacobs, 1978). Similar to β-haemolysin, the GBS pigment was shown to be released into the culture medium using starch (Merritt & Jacobs, 1978; Tapsall, 1987; Wennestrom *et al.*, 1991).

The chemical structure of the chromophore in the GBS pigment was elucidated using NMR spectroscopy and MS. It was shown to be an ornithine rhamnopolyene named granadaene with a linear chain of 12 unsaturated conjugated bonds (Rosa-Fraile *et al.*, 2006; Paradas *et al.*, 2012) (Fig. 2). The granadaene molecule exhibits some characteristics of an acid–base indicator, and its UV–visible absorption spectrum shifts from a carotene-like spectrum, with three peaks at 525, 485 and 455 nm at low pH (red colour), to a one-peak spectrum of 420 nm (yellow colour) at high pH (Rosa-Fraile *et al.*, 2006). Under certain conditions, for example in the presence of amylase or serum, GBS produces a one-peak pigment, which could explain previous reports suggesting the existence of two different pigments (Tapsall, 1986, 1987; Haug & Soderlund, 1977). However, it remains to be determined whether granadaene represents the full GBS pigment. It is possible that an additional part that attaches the pigment to the cell wall was removed during the stringent conditions [dimethylsulfoxide – trifluoroacetic acid (DMSO-TFA)] used for pigment extraction (Rosa-Fraile *et al.*, 2006).

GBS pigment is a polyene, and the polyene biosynthesis pathway closely resembles that of fatty acid biosynthesis (Schweizer, 1989; Goel *et al.*, 2002). The *cyl* chromosomal locus encoding haemolysin and pigment production includes several genes (*cylD*, *cylG*, *cylZ*, *apcC* and *cylI*) with homology to enzymes involved in fatty acid – and polyene – biosynthesis (Goel *et al.*, 2002; Aparicio *et al.*, 2004). Based on these homologies, a theoretical pathway for the biosynthesis of GBS pigment has been suggested (Whidbey *et al.*, 2013; Fig. 2).

Inhibitors of the folate pathway trigger pigment production in human GBS strains (Rosa *et al.*, 1983, 1992; Schaufuβ *et al.*, 1985; Tapsall, 1987). Methylfolate is a key intermediate in the biosynthesis of thymidine and is the carrier of the hexose in the biosynthesis of rhamnolipids (Pazur & Shuey, 1961; Burger *et al.*, 1963; Ochsner *et al.*, 1994). CylJ displays homology to a glycosyltransferase (Pritzlaff *et al.*, 2001) and presumably encodes the rhamnosyltransferase used in the biosynthesis of granadaene. However, the theoretical biochemical pathway proposed for the biosynthesis of granadaene (Whidbey *et al.*, 2013; Fig. 2) does not account for the pigment-enhancing effect of folate inhibitors. Interestingly, the pigment-enhancing effect of folate antagonists is not seen in most bovine strains (Schaufuβ *et al.*, 1985). This fact, together with the lack of pigment production in a high proportion of these strains (Mhalu, 1976; Merritt & Jacobs, 1978; Brglez, 1983; Lämmler *et al.*, 1985; Garcia *et al.*, 2008), prevents the use of Granada media (Rosa *et al.*, 1992) for the detection of bovine GBS infections.

GBS haemolysin and pigment

More than 30 years ago, GBS pigment and haemolysin were identified as key determinants for GBS pathogenicity. A close link between these two phenotypic traits has been reported for some time but has never been fully explained, until a recent study demonstrating that GBS haemolysin and pigment appear to exhibit, Janus-like, the two faces of a single virulence factor.

Phenotypic linkage of GBS haemolysin and pigment production

A strong link exists between the β-haemolytic phenotype and the production of GBS pigment (Lancefield, 1934; Sherman, 1937; Plummer, 1941). Systematic studies could not identify nonhaemolytic pigmented GBS strains or nonpigmented haemolytic strains (Fallon, 1974; Noble *et al.*, 1983; Tapsall, 1987; Wennestrom *et al.*, 1991). Moreover, the amount of pigment produced by GBS wild-type isolates always correlates with the amount of haemolysin produced (Wennerstrom *et al.*, 1985; Tapsall, 1987; Nizet *et al.*, 1996). Nonhaemolytic mutants isolated from a large GBS mutant library were mapped in the *cyl* operon and were simultaneously altered in pigment production (Wennerstrom *et al.*, 1985; Nizet *et al.*, 1996; Spellerberg *et al.*, 1999, 2000a; Forquin *et al.*, 2007; Pritzlaff *et al.*, 2001). As a proof that haemolysin and pigment activities are carried by the same molecule, it has been shown that a nonhaemolytic preparation of GBS pigment displays haemolytic activity after the addition of starch (Whidbey *et al.*, 2013).

Pigmentation and haemolysis in veterinary GBS strains

In veterinary GBS isolates nonpigmented strains are frequently reported in addition to a lack of correlation between pigment and haemolysin production (Mhalu, 1976; Islam, 1977; Merritt & Jacobs, 1978; Brglez, 1983; Lämmler *et al.*, 1985; Garcia *et al.*, 2008). This discrepancy may have been caused by the diversity of culture media used to detect pigment and haemolysin (Merrit *et al.*, 1976; Mhalu, 1976; Lämmler *et al.*, 1985), given that media lacking proteose peptone 3 and folate pathway inhibitors (Rosa-Fraile *et al.*, 1999a) may lead to an overestimation of nonpigmented strains. Therefore, a careful re-evaluation of these findings using quantitative methods, standard media for the detection of GBS pigment (e.g. Granada medium) and sequencing of the *cyl* gene cluster may solve previously reported discrepancies between pigment and haemolysin production.

Moreover, variations in the *cyl* gene cluster were reported in recently sequenced GBS strains from fish.

Some strains harbour the complete *cyl* gene cluster and display β-haemolysis, while other strains are nonhaemolytic and contain mutations in the *cyl* locus (Liu *et al.*, 2012, 2013; Pereira *et al.*, 2013). Analysis of the nonhaemolytic STIR-CD-17 GBS strain isolated from tilapia (*Oreochromis* sp.) indicated that only parts of the *cyl* operon are present in this strain (Delannoy *et al.*, 2012). Interestingly in another nonhaemolytic GBS strain isolated from tilapia an incomplete *cyl* operon was found containing *cylE*, *cylA* and *cylB* (Liu *et al.*, 2013; Pereira *et al.*, 2013), thus supporting the interpretation that these genes are necessary but not sufficient for GBS haemolysis. In fish pathogenic strains, most hypervirulent isolates are nonhaemolytic, indicating that the β-haemolysin is not an important virulence factor in these hosts (C.M.J. Delannoy, pers. commun.). Genome analysis of veterinary GBS strains reveals a considerable heterogeneity and shows that mechanisms of acquisition, duplication and reshuffling have permitted GBS to adapt to different environmental niches (Tettelin *et al.*, 2005; Delannoy *et al.*, 2012; Liu *et al.*, 2012; Pereira *et al.*, 2013; Rosinski-Chupin *et al.*, 2013; Wang *et al.*, 2013; Zubair *et al.*, 2013).

The role of GBS haemolysin and pigment in virulence

Haemolysin

GBS, a commensal bacterium that asymptomatically colonizes human mucosal surfaces, can turn into a life-threatening pathogen in susceptible hosts (Ewards & Baker, 2010; Rodriguez-Granger *et al.*, 2012). The molecular bases underlying GBS infections have been unveiled in the last decade and several reviews described the identification and importance of these virulence factors (Doran & Nizet, 2004; Nizet & Rubens, 2006; Maisey *et al.*, 2009; Rajagopal, 2009).

Among them, the cell surface-associated β-haemolysin is thought to play a key role by promoting GBS penetration of host cell barriers such as the epithelial and endothelial cells of the lung and the blood–brain barrier. Furthermore, β-h/c was shown to induce host inflammatory responses (Doran *et al.*, 2002; Bebien *et al.*, 2012; Costa *et al.*, 2012). The membrane-damaging effect of β-haemolysin is not restricted to erythrocytes; GBS β-haemolysin extracts exert a direct cytotoxicity against different eukaryotic cell types (Liu *et al.*, 2004; Hensler *et al.*, 2008a; Alkuwaity *et al.*, 2012). Different studies suggest that GBS β-haemolysin has immunomodulatory properties that favour intracellular survival of GBS contributing to virulence (Doran *et al.*, 2002; Liu *et al.*, 2004; Bebien *et al.*, 2012). Haemolysin-deficient mutants are attenuated in virulence in several animal models of GBS

infections. By contrast, hyperhaemolytic GBS strains exhibit increased virulence (Wennerstrom *et al.*, 1985; Tapsall & Phillips, 1991; Wennerstrom *et al.*, 1991; Nizet *et al.*, 1996, 1997b; Gibson *et al.*, 1999; Puliti *et al.*, 2000; Doran *et al.*, 2002, 2003; Ring *et al.*, 2002; Liu & Nizet, 2004; Hensler *et al.*, 2005, 2008a; Forquin *et al.*, 2007; Kaplan *et al.*, 2008; Lembo *et al.*, 2010; Fettucciari *et al.*, 2011; Alkuwaity *et al.*, 2012; Bebien *et al.*, 2012; Whidbey *et al.*, 2013). In addition, strong haemolytic GBS strains but not weak or nonhaemolytic strains are able to trigger macrophage apoptosis and to disrupt the macrophage cytoskeleton (Fettucciari *et al.*, 2000, 2006, 2011; Liu *et al.*, 2004; Liu & Nizet, 2006; Whidbey *et al.*, 2013). Hyperhaemolytic GBS strains have been reported to be more frequently associated with women in preterm labour (Whidbey *et al.*, 2013) and with cases of GBS streptococcal toxic shock syndrome and necrotizing fasciitis (Sendi *et al.*, 2009). In line with these findings, it has also been reported that GBS nonhaemolytic strains are quite infrequent among GBS strains causing neonatal infections (Rodriguez-Granger *et al.*, 2011). Moreover, the effect of intravenous administration of partially purified haemolysin in rabbits or rats produced dose-dependent hypotensive changes and deaths due to shock (Nizet *et al.*, 1996). Nevertheless, controversial reports exist about the exact role of β-haemolysin in GBS infections (Sendi *et al.*, 2009; Cumley *et al.*, 2012; Sagar *et al.*, 2013). Two reports indicate no differences between haemolytic and their nonhaemolytic counterparts in a mouse or a neonatal rat sepsis model (Wennerstrom *et al.*, 1985; Weiser & Rubens, 1987). Surprisingly, GBS strains belonging to the hypervirulent clonal complex 17 that are responsible for the majority of invasive neonatal infections in Europe (Poyart *et al.*, 2008; Fluegge *et al.*, 2011) exhibit very low levels of haemolysis (Tazi *et al.*, 2012). However, it remains possible that the amount of β-haemolysin production observed *in vitro* may not reflect the *in vivo* situation.

Pigment

It was first suggested in the 1980s that the pigment of GBS can neutralize superoxide and therefore pigment could confer resistance to radical oxygen species (Nemergut & Merritt, 1982, 1983). Indeed, it has been shown that a filtered extract of GBS pigment confers the ability to resist to the antimicrobial effects of reactive oxygen species during phagolysosomal killing (Liu & Nizet, 2004; Liu *et al.*, 2004). Nevertheless, these studies assumed that GBS pigment was a carotene, whereas it is a polyene (nonisoprenoid) (Rosa-Fraile *et al.*, 2006). Polyenic pigments share with isoprenoid carotenes a conjugated double-bond system with delocated electrons resulting in a characteristic carotene-like UV–visible spectrum (Britton,

1995). Polyenic pigments can also act – as do carotenes – as antioxidants (Krinsky, 1979; Krinsky & Yeum, 2003) and can protect membrane lipids against peroxidation, as described for some plant pathogenic bacteria, i.e. xantomonadin in *Xantomonas orizae* (Rajagopal *et al.*, 1997; Goel *et al.*, 2002). A recent study reported no difference in survival within mouse monocyte-derived macrophages between a nonpigmented *cylE* mutant and its wild-type counterpart, challenging the idea that pigment is a crucial virulence factor for resisting macrophage killing (Cumley *et al.*, 2012).

Other bacterial components displaying similarity to GBS haemolysin and granadaene

While the GBS granadaene is unique among streptococci, it has been found in other bacterial species. *Propionibacterium jensenii* is a gram-positive, high-G + C-content bacterium that causes splitting and formation of red spots in Swiss-type cheeses. *Propionibacterium jensenii* is β-haemolytic and produces a red pigment identical to GBS granadaene. A genetic link between pigmentation and haemolytic activity in *P. jensenii* was suggested by chemical mutagenesis studies using nitrosoguanidine. Nonpigmented mutants of *P. jensenii* are nonhaemolytic, while mutants demonstrating a reduced pigmentation also display a diminished haemolytic activity (Vanberg *et al.*, 2007). Pigment is also invariably linked with β-haemolysis in other species of *Propionibacterium*, such as *P. thoenii* and *P. rubrum* (Vedamuthu *et al.*, 1971). As for *P. jesenii*, chemical mutagenesis in *P. thoenii* causes a simultaneous loss of haemolysis and pigment production (Vanberg *et al.*, 2007). Nevertheless, to the best of our knowledge, the production of granadaene in *P. rubrum* and *P. thoenii* has not been investigated. Granadaene has been detected in *P. jensenii*, and it is interesting to note that a gene cluster with considerable similarities to the *cyl* genes has been identified in this bacterium. With the exception of *cylK*, homologues of all of the *cyl* genes are present in *P. jensenii*, but with a different gene organization (C. Vanberg C., Langsrud T., Nes I.F. & Holo H, unpublished data, *P. jensenii* strain LMG 2818 granadaene gene cluster, complete sequence GenBank: FJ617193.1).

With the increasing availability of bacterial genomes, orthologues and paralogues of the *cyl* gene cluster could be identified in a number of bacterial species distantly related to streptococci such as *Bacillus cereus*, *propionibacterium* spp., *Arthrobacter aurescens* and *Actinomyces viscosus* (Whidbey *et al.*, 2013). Interestingly, all these species are gram-positive rods, some as in the case of *Arthrobacter* spp. are frequently found in environmental soil samples and these species do not represent classical

human pathogens. This observation may indicate a function of the *cyl* genes that is not linked to human pathogenesis.

GBS pigment and haemolysin, identical or nonidentical twins?

Because of the close link between haemolysin and pigment production, it has been suggested that the pigment could be the natural carrier for the haemolysin or that GBS pigment and haemolysin are identical (Tapsall, 1987; Pritzlaff *et al.*, 2001; Liu & Nizet, 2006; Whidbey *et al.*, 2013). The addition of starch to a nonhaemolytic GBS pigment solution in DMSO-TFA results in a haemolytic and cytolytic preparation (Whidbey *et al.*, 2013) leading to the conclusion that GBS pigment and haemolysin are identical molecules. Nevertheless, it remains possible that – although closely related – pigment and haemolysin could be slightly different molecules. For example, the pigment dissolved in DMSO-TFA and the haemolytic mixture of pigment and starch are stable, in contrast to the poor stability of GBS haemolysin–starch preparations (Dal & Monteil, 1983), and haemolytic colonies of GBS on blood agar plates are not pigmented. Different culture conditions can result in different haemolysin and pigment production (Rosa-Fraile *et al.*, 1999a; Tapsall, 1987). Many early studies reporting differences between haemolysin and pigment production relied on qualitative visual detection of haemolysin and pigment, and this may explain the observed discrepancies between pigment and haemolysin to some extent. Some reports indicate that trimethoprim increases pigment production but does not have a stimulatory effect on haemolysin production (Schaufuβ *et al.*, 1985; Tapsall, 1987). However, in contrast to these reports, on blood agar plates inoculated with a haemolytic GBS strain and with a sulfamethoxazole/trimethoprim (23.75/1.25 μg) antibiotic paper disc, an increased haemolysin production occurs in the GBS colonies surrounding the antibiotic disc (Fig. 4). This observation raises doubts about the validity of previous reports indicating that folate pathway inhibitors do not increase GBS haemolysin production. It is also worth pointing out that GBS pigment is stabilized by the same carrier molecules – albumin and starch – that are required to stabilize haemolysin (Tapsall, 1986), and both carrier substances can closely bind to long-chain fatty acids (Mikus *et al.*, 1946; BeMiller, 1965; Spector *et al.*, 1969; Spector, 1975; Blazek, 2008).

Polyenes display a high affinity to sterols and phospholipids of the cell membrane, and they are haemolytic as well as cytolytic (Bolard, 1986; Knopik-Skrocka & Bielawski, 2002; Aparicio *et al.*, 2004; Knopik-Skrocka *et al.*, 2007). The toxicity of polyenes against mammalian cells
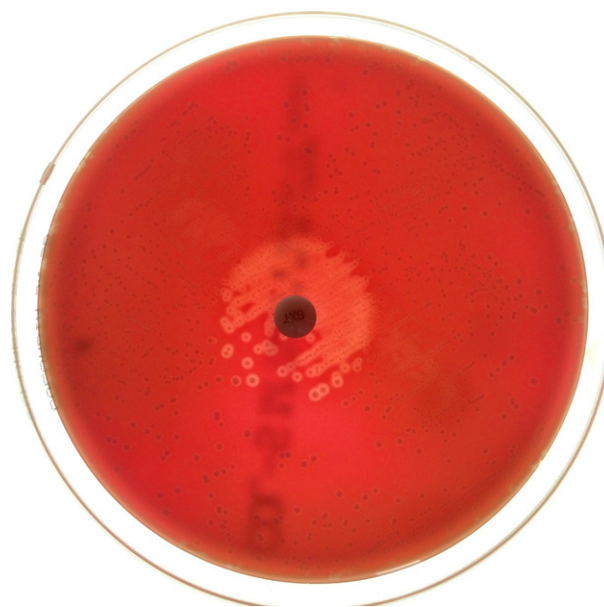
Fig. 4. Enhancing effect of folate inhibitors on GBS haemolysin. Depicted is a picture of a blood agar plate inoculated with a haemolytic GBS strain and with a sulfamethoxazole/trimethoprim (23.75/1.25 μg) antibiotic paper disc.

is well documented in polyenic antibiotics (Bolard, 1986), and cytotoxicity is observed even in polyene molecules harbouring only four conjugated double bonds (Bae *et al.*, 2013). The formation of polyene–lipid complexes can lead to changes in membrane permeability, resulting in haemolysis and cytolysis (Hsuchen & Feingold, 1973; Siegel, 1977; Brajtburg *et al.*, 1980; Bolard, 1986; Aparicio *et al.*, 2004). However, cholesterol, which inhibits the haemolytic activity of some polyenic antibiotics (Hsuchen & Feingold, 1973; Strom *et al.*, 1979), had no inhibitory effect on the GBS haemolysin that is inhibited by phospholipids (Marchlewicz & Duncan, 1980; Ferrieri, 1982; Tapsall & Phillips, 1991).

It is also possible that the rhamnose tail of the GBS pigment may contribute to its membrane-damaging activity, as bacterial di-rhamnolipids are haemolytic. This haemolytic activity has been attributed to their biosurfactant characteristics as well as their cone-shaped configuration (Abdel-Mawgoud *et al.*, 2010; Ortiz *et al.*, 2010; Sanchez *et al.*, 2010). While there appear to be structural differences between di-rhamnolipids and granadaene, it is possible that the rhamnose tail of GBS pigment may play a role in the membrane-damaging effect. The available data strongly support the hypothesis that GBS haemolysin and GBS polyenic pigment share a common metabolic pathway encoded in the *cyl* operon and that the haemolysin is a molecule closely related or identical to the granadaene polyenic pigment.

Summary

Research on the GBS β-h/c and pigment, two major phenotypic traits used for bacterial identification, has been being conducted for almost a century. GBS harbour a unique set of 12 genes, the *cyl* operon, responsible for both haemolysin and pigment production. Expression of the *cyl* operon is controlled by the CovS/R two-component system, which coordinates the expression of multiple virulence factors. Several publications have shown the important role of β-h/c in virulence. However, due to its small molecular size, the apparent lack of immunogenicity and a rapid loss of activity, the biochemical nature of the GBS β-h/c has remained elusive for many years. In 2000, the *cyl* operon was shown to be responsible for haemolysin and pigment production, and in 2006 the structure of the pigment was solved and demonstrated to be an ornithine-rhamnolipid designated 'granadaene'. Recently, this rhamnolipid was shown to be haemolytic under certain experimental conditions. These data strongly support the idea that the GBS β-h/c and pigment are identical or very closely related molecules. Purification of β-h/c and determination of its structure will definitely prove this hypothesis.

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