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Roles of environmental heme, and menaquinone, in *Streptococcus agalactiae*

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Abstract

Most bacteria require iron for growth. However, as it may not be directly available under aerobic conditions, bacteria may use iron-sequestering molecules, such as bacterially encoded siderophores, or heme, which is the major iron source in the animal host. Bacteria may also assimilate heme for purposes other than as an iron source. Once internalised, heme can activate, for example, a heme-dependent catalase and a cytochrome oxidase. In bacterial pathogen *Streptococcus agalactiae*, heme, in association with exogenous menaquinone, activates a respiratory chain. Respiration has radical effects on carbon metabolism. GBS respiration-grown cells display improved survival in an aerobic environment and greater virulence in a murine septicemia model. GBS might benefit from its ecological niches to capture heme and menaquinone, i.e., from other bacteria when it colonizes host mucosa, or from blood-containing organs during septicemia.

Different forms of iron

Iron is used as cofactor in key enzymes like aconitase, cytochrome oxidase, or aerobic ribonucleotide reductase. This metal is thus considered as an essential nutrient for growth of most, if not all bacteria (Ratledge & Dover 2000). However, under oxygen and at physiological pH, free ferrous iron is rapidly oxidized to insoluble ferric hydroxide. Solubility of $Fe(OH)_n$ is around 10^{-9} M, which is below the concentration estimated to support bacterial growth ($>10^{-7}$ M) (Ratledge & Dover 2000). To avoid iron starvation and to assess their growth, a majority of bacteria secrete into the medium siderophores (Ratledge & Dover 2000), which have a strong affinity for ferric iron. The iron-siderophore complex is soluble and can be easily taken up by bacteria via receptors present in outer membranes of Gram-negative bacteria, or cytoplasmic membranes of Gram-positive bacteria (Wandersman & Delepelaire 2004). Note that the ferrous iron, which is soluble in water, can be directly transported into cells without siderophores. Interestingly, some bacteria do not produce siderophores, although they do have an obligate iron requirement (Evans *et al.* 1986; Tai *et al.* 1993). To capture iron, these bacteria could use siderophores produced by other bacteria (Sebulsky *et al.* 2000) or steal iron from metalloproteins (Eichenbaum *et al.* 1996; Rouault 2004).

In human fluids, iron is sequestered by glycoproteins such as lactoferrin, which complexes the ferric form ($K_d = 10^{-20}\ M^{-1}$). However, more than 80% of iron is present in hemoproteins (Skaar *et al.* 2004). Heme is mainly found in red blood cells or in specific organs like the liver. In

addition, during turnover of red blood cells, the degradation of hemoglobin liberates heme, which is further carried out by carriers like hemopexin or serum albumin (Wandersman & Delepelaire 2004). These proteins deliver heme to the liver where it is degraded. This mechanism of heme-iron carriage in human fluid probably avoids both the strong toxicity of free heme (Aft & Mueller 1983, 1984; Stojiljkovic & Hantke 1994) and limits the availability to bacterial pathogens of heme-iron in the blood stream.

Heme capture

In Gram-negative bacteria, receptors in the outer membrane are in direct contact with the environment. Receptors recognise different forms of heme, including the free heme molecule, or complexed heme (e.g., associated with hemopexin or hemoglobin) with specificities that vary with the strain. For instance, the *Yersinia enterocolitica* heme receptor (HemR) recognizes a large spectrum of hemoproteins (hemopexin, hemoglobin, and catalase) while the hemoglobin receptor (HmbR) has a strong preference for heme and hemoglobin but not for catalase or hemopexin (Bracken *et al*. 1999). After the binding step, heme passes through the outer membrane by a TonB-ExbB-ExbD-dependent mechanism. Furthermore, heme is internalised into cytoplasm by transporters belonging to the ABC family which requires ATP as energy.

In contrast to Gram-negative bacteria, heme acquisition systems used in Gram-positive bacteria have been less characterized (Brown & Holden 2002). Heme-binding proteins have been identified but their exact roles in heme uptake remain to be clarified. Some ABC-type transporters were shown to transport heme, and are strongly similar to those in Gram-negative bacteria except that the receptor is a lipoprotein (Schmitt 1997; Skaar *et al*. 2004; Tai *et al*. 2003). However, this raised several questions: (i) Does the receptor directly recognize hemoproteins, or is the protein first degraded to liberate heme? (ii) How do Gram-positive bacteria resolve the steric problem of heme receptor access to its substrate across capsule, techoic acid, and peptidoglycan barriers? Elegant work on *Staphylococcus aureus* from the Schneewind laboratory identified cell wall-anchored proteins (IsdA, IsdB, IsdC) needed for heme capture from hemoprotein (Mazmanian *et al*. 2003). The genes responsible for heme transport are organised in one cluster *isdB-isdA-isdC-isdD-isdE-isdF-srtB-isdG*. The proteins, IsdA and IsdB, contain the LPXTG motif, which is cleaved by Sortase A and covalently linked to peptidoglycan. In contrast IsdC contains a derived motif, NPQTN, but is covalently linked to peptidoglycan via a second sortase SrtB. By homology, IsdA and IsdB are potential heme receptors, while IsdC, which is buried in the cell wall, probably shuttles heme from the surface to IsdE, a lipoprotein associated with the IsdD-IsdF permease. Recently, a similar system has also been reported in *Lysteria monocytogenes* (Newton *et al*. 2005) indicating that this mechanism of iron transport from cell surface to membrane may be used in different Gram-positive bacteria. Interestingly, another heme carrier system was identified in *Streptococcus pyogenes* (Liu & Lei 2005). In this bacterium, heme-binding protein Shp does not contain the LPXTG motif and is likely to not be covalently linked to cell wall (Lei *et al*. 2002). Nevertheless, Shp transfers heme to HtsA, a lipoprotein of the HtsABC transport system. In addition, the *shp* gene is organised in an operon with *htsABC*, consistent with a role for Shp as a heme carrier.

Heme as a source of heme, rather than of iron

In several bacteria, internalised heme is degraded by a heme oxygenase to biliverdin and carbon monoxide, using NAD(P)H, to liberate iron. This enzyme has been characterized in pathogen bacteria, confirming the importance of iron for growth (Schmitt 1997; Wu *et al*. 2005; Zhu *et al*. 2000). However, in some Streptococcaccae, such as *Lactococcus lactis* and *Enterococcus faecalis*, iron was reportedly dispensable for growth (Duwat *et al*. 1995; Marcelis *et al*. 1978; Pandey *et al*. 1994). Nevertheless, both these bacteria incorporate heme, and use it to activate hemoproteins like catalase (Frankenberg *et al*. 2002), or cytochrome oxidase (Duwat *et al*. 2001; Ritchey & Seely 1976; Sijpesteijn 1970; Winstedt *et al*. 2000; Yamamoto *et al*. 2005). Thus, while free iron might be liberated from heme in these bacteria by unknown mechanisms, it was clearly shown that heme is used directly to activate heme-requiring enzymes.

Interestingly, cytochrome oxidases described in *E. faecalis* (Winstedt *et al.* 2000), in *L. lactis* (Duwat *et al.* 2001) and more recently in *Streptococcus agalactiae* (Yamamoto *et al.* 2005) belong to the bd-cytochrome oxidase family coded by *cydAB* genes.

Heme and *Streptococcus agalactiae*

S. agalactiae (also referred to as Group B Streptococcus, or GBS) is a Gram-positive bacterium belonging to the Streptococcaccae family including Streptococcus, Lactococcus, Enterococcus. Fermentation has been described as its only energy mode in GBS. GBS is a part of normal gastrointestinal and vaginal flora where colonization is asymptomatic. However, GBS is a leading cause of invasive infection among newborns but also in immunocompromised adults and in the elderly (Schuchat 1999). In addition, GBS is also responsible of bovine intramammary infection (mastitis). The main route of infection in neonates is assumed to be via aspiration of the vaginal content or the amniotic fluid containing GBS during delivery, which are then spread, leading in 2% of the cases to sepsis and meningitis. Several factors have been characterized as contributing to the success of GBS infection and they are mainly extracellular elements, e.g., capsule, adhesins, hemolysins, and proteases (Lindahl *et al.* 2005). Only few examples of intracellular factors have been described, e.g., involving purine metabolism, methionine transport, and MnSOD (Poyart *et al.* 2001; Rajagopal *et al.* 2005; Shelver *et al.* 2003).

To date, the role of iron and heme in GBS virulence has not been clearly addressed, although studies have shown that hemolysin CylE might contribute to heme capture (Lamy *et al.* 2004; Liu *et al.* 2004; Weiser & Rubens 1987). Genome analysis of two GBS strains (NEM316 and 2603V/R) reveals the presence of *cydAB* genes, which encode the structural components of the terminal cytochrome oxidase that constitutes a part of respiratory chain. *cydAB* genes are located in an operon including two downstream genes coding permease subunits, CydC (Gbs1785) and CydD (Gbs1784) (Glaser *et al.* 2002; Tettelin *et al.* 2002). In *E. coli*, the CydCD complex is involved in CydAB maturation probably by transporting heme or cysteine (Pittman *et al.* 2002). Just upstream of the *cyd* genes, *gbs1788* and *gbs1789*, another respiratory related genes, are located. *gbs1788* encodes a putative NADH dehydrogenase similar to the *E. coli* Ndh-II; Ndh transfers electrons from NADH to menaquinone, which in turn shuttles electrons to cytochrome oxidase. *gbs1789* encodes a MenA/UbiA homologue, which putatively catalyses the last step in menaquinone synthesis. Unlike the case in *E. coli*, the menaquinone biosynthesis pathway in GBS is incomplete (so is the heme biosynthesis pathway). Thus, this operon seems to encode all the proteins needed to constitute a complete respiratory chain, with the exception of two cofactors, heme and menaquinone.

Evidence for respiration capacity in GBS

The ability of GBS to disseminate in blood and the presence of potential respiratory genes led us to hypothesize that it could take up heme to activate its cytochrome oxidase. To determine the respiration capacity, cell density and pH, which are hallmarks of respiration/fermentation metabolism, were measured in rich medium (M17) supplemented with glucose as carbon source. Heme alone, or menaquinone alone, was not sufficient to trigger respiration under aerobic condi-

Table 1. Effects of heme and menaquinone (MQ) on aerobic growth of GBS.

Growth conditions	Biomass (g/l)	pH	$[O_2]$(μM/min)	NADH oxidase (units/mg)
O_2	1.4	4.8	25	0.03
O_2, Heme or MQ	1.4	4.8	n.d.	n.d.
O_2, Heme and MQ	2.1	5.6	53	0.55
O_2, Blood and MQ	n.d	n.d.	46	0.3

The menaquinone (MQ) used was menatetrenone (MK_4). Biomass and pH of medium were measured after overnight growth. Oxygen consumption and NADH oxidase activity were measured in resting cells and in membrane fractions, respectively, as described (Yamamoto *et al.* 2005).

tions (Table 1). However, the combined addition of heme and menaquinone led to enhanced growth and higher final pH of the medium. These gains are correlated with an increase of oxygen consumption in resting cells and a stronger NADH oxidase activity in the membrane fraction. Moreover, a known specific inhibitor of cytochrome oxidase, 2-heptyl-4-hydroxyquinoline, abolished the gain of biomass observed under heme and menaquinone conditions. Taken together, these results confirm the respiration capacity of GBS, respiration that is activated by two exogenous compounds: heme and menaquinone. Biochemical analysis of glucose degradation products indicated that a part of the pyruvate pool is deviated to acetate and acetoin at the expense of lactate, which is the major fermentation end product. In addition, real-time PCR results shown that *cydA* expression was 3- to 5-fold increased late in growth and whatever the growth conditions (static, aeration, or heme plus menaquinone) (Yamamoto *et al*. 2005).

Evidence for a role of respiration in virulence

GBS respiration requires heme and menaquinone, both of which could be provided by host blood (menaquinone concentration is estimated at 2 ng/ml in rat blood), or by blood-rich organs. In addition, GBS respiration resulted in effective energy gain and improved bacterial survival *in vitro* compared with fermentation (Yamamoto *et al*. 2005). These results led us to ask whether respiration contributes to virulence. To determine the role of respiration in GBS virulence, we constructed *cydA* mutants; one contained a kanamycin cassette insertion, and the other, a single amino acid substitution (tryptophan to alanine at position 391; cydA^{W391}) in the highly conserved catalytic domain of CydA proteins (GWXXXEXGRQPW) (Zhang *et al*. 2001). As expected, both mutations abolished the respiration capacity, and mutants grew only via fermentation metabolism *in vitro*. Virulence of GBS wild type strain NEM316 and these respiration-negative strains were further tested in a neonatal rat sepsis model (Figure 1). The wild type strain killed almost all rats within three days, while *cydA* mutants were less virulent. These effects were observed regardless of the way that GBS culture was prepared (static, aerobic or respiration conditions). These results showing that CydA is crucial for virulence, point to a role for respiration metabolism in the GBS infection process.

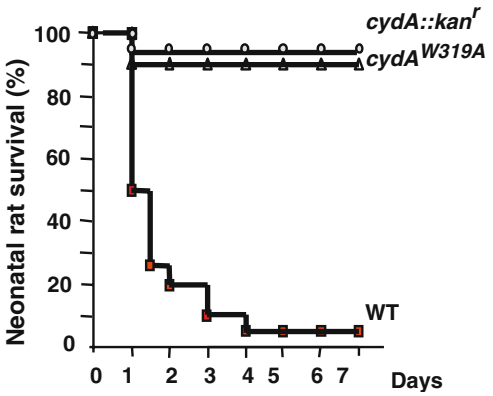

Figure 1. Mortality curves in neonates rats infected intraperitonally with 5×10^6 cfu of wild type strain or *cydA* mutants.

Conclusion & perspectives

Bacteria possess numerous means to capture iron from iron-containing complexes or directly assimilate ferrous iron, as described mainly in Gram-negative bacteria. Receptors and permeases have been identified, and for some bacteria, the mechanism of transport has been characterized. In GBS, an iron requirement remains to be demonstrated. However, we showed that heme capture is used and activates a cytochrome oxidase, which in combination with exogenous menaquinones, completes the electron transport chain for respiration. Thus, heme seems to be used directly, rather than as a free iron source. To date, GBS heme receptors have not been identified by experimentation based on previously characterised heme-binding proteins in other bacteria. Identification of the active GBS heme receptors is in progress in our laboratory.

cydAB genes coding bd-cytochrome oxidase are regulated late in growth in several bacteria and oxygen level was incriminated as signal of regulation (Cotter *et al*. 1990; Kana *et al*. 2001; Wu *et al*. 1997). However, it is unlikely that this is the case in GBS since even under static conditions *cydA* gene was up-regulated (Yamamoto *et al*. 2005). Recently, a redox regulator (Rex) of *cydAB* genes has been described in *Streptomyces coelicolor*, (Brekasis & Paget 2003). As binding to DNA is affected

by NAD(H), authors proposed that NADH/NAD ratio might be a signal for Rex. Rex-like proteins are highly conserved among Gram-positive bacteria; a homologue in *Bacillus subtilis* was shown to control *cydAB* expression Schau *et al*. 2004), and similar Orfs are present in genomes of *L. lactis* (YkhD, (Bolotin *et al*. 2001)), of *E. faecalis* V583 [EF2638, EF2933, (Paulsen *et al*. 2003)], and of GBS [Gbs1167, (Glaser *et al*. 2002)].

Heme and menaquinone, present in blood and blood-rich organs, are essential for GBS respiration. *cydA* inactivation attenuated GBS virulence in a murine septicemia model. Thus respiration, and/or respiration-controlled functions, may contribute to dissemination of GBS in the animal or human host. Respiration metabolism constitutes an energy gain for the bacterium, and may also help eliminate oxygen species from its immediate environment. The shift to respiration metabolism may also result in profound changes in gene expression, including that of virulence factors. Our future studies will be aimed at determining what aspects of GBS respiration contributes to its virulence.

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