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Laétitia Joubert, Aurelie Bobillot, Philippe Gaudu, Alexandra Gruss, Delphine Lechardeur. HrtBA and menaquinones control haem homeostasis in *Lactococcus lactis*. *Molecular Microbiology*, 2014, 93 (4), pp.823 - 833. 10.1111/mmi.12705 . hal-01204405

HAL Id: hal-01204405

<https://hal.science/hal-01204405v1>

Submitted on 27 May 2020

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HrtBA and menaquinones control haem homeostasis in *Lactococcus lactis*

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Summary

Lactococcus lactis is a fermenting Gram-positive bacterium widely used for production of dairy products. Lacking haem biosynthesis genes, *L. lactis* can still shift to an energetically favourable respiratory metabolism by activating a terminal cytochrome *bd* oxidase when haem is added to an aerated culture. Haem intracellular homeostasis is mediated by the *hrtRBA* operon encoding the conserved membrane HrtBA haem efflux permease and the unique intracellular haem sensor and regulator, HrtR. Here we report that membrane-associated menaquinones (MK) favour the accumulation of reduced haem in membranes. An oxidative environment, provided by oxygen, prevents and reverses haemin reduction by MK and thus limits haem accumulation in membranes. HrtBA counteracts MK-dependent membrane retention of excess haem in membrane, suggesting direct efflux from this compartment. Moreover, both HrtBA and MK-mediated reduction have a strong impact on haem intracellular pools, as determined via HrtR haem sensor induction, suggesting that intracellular haem acquisition is controlled at the membrane level without the need for dedicated import systems. Our conclusions lead to a new hypothesis of haem acquisition and regulation in which HrtBA and the bacterial membrane have central roles in *L. lactis*.

Introduction

Haem and respiration in Lactococcus lactis

Haem, an iron-containing porphyrin, is the prosthetic group for numerous key cellular enzymatic and regulatory

processes (Furuyama *et al.*, 2007; Gruss *et al.*, 2012; Girvan and Munro, 2013). The porphyrin ring facilitates iron solubility and optimizes iron catalytic activity by 5–10 orders of magnitude (Kraemer, 2004). The properties of iron as a transition metal that cycles between redox states are maintained in haem, making it a valuable cofactor for biological processes, but generating toxic free radicals through Fenton chemistry (Alayash *et al.*, 2001). The resulting reactive oxygen species can be deleterious to lipids, proteins and nucleic acids.

Most bacteria encode the biosynthetic enzymes needed for endogenous haem production. Remarkably however, numerous bacteria lack a complete haem biosynthesis pathway, yet encode haem-requiring functions. For such haem-auxotrophs, haem must be captured from the environment for direct use. This is in contrast to most studied bacteria, which synthesize haem but also scavenge it from their environment to use as an iron source (Anzaldi and Skaar, 2010; Pishchany and Skaar, 2012). A well-studied example of a haem auxotroph is *Lactococcus lactis*, a fermenting bacterium that produces lactic acid, which can shift to an energetically favourable respiratory metabolism when haem is added to an aerated culture. Respiration metabolism has a major positive impact on growth and long-term survival (Duwat *et al.*, 2001; Lechardeur *et al.*, 2011; Pedersen *et al.*, 2012). Numerous other lactic acid bacteria (LAB), including opportunist pathogens like *Streptococcus agalactiae* and *Enterococcus faecalis* similarly use haem to activate respiration growth (Winstedt *et al.*, 2000; Yamamoto *et al.*, 2005; Gruss *et al.*, 2012). Haem-mediated respiration in *L. lactis* depends on endogenously synthesized membrane-localized lipophilic menaquinones (MK), which shuttle electrons from oxidoreductases to cytochrome oxidases (Gaudu *et al.*, 2002; Rezaiki *et al.*, 2004). In bacteria, quinones also accept electrons from proteins other than dehydrogenases, like redox proteins, and participate in multiple cellular functions such as secretion and defence against oxidative stress (Nohl *et al.*, 2003; Yamamoto *et al.*, 2006). MKs synthesized by *L. lactis* consist of two distinct subgroups: (i) MK-8 to MK-10, considered as hallmarks of *L. lactis*, are produced throughout growth, and (ii) Short-chain MK-3 and demethyl MK-3 are present only in early stationary growth phase (Rezaiki *et al.*, 2004). Quinone profiles were similar for extracts derived from static, aeration or respiration (in the presence of haemin and oxygen). The redox state of MKs varies

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according to oxygen and haem availability, and cytochrome *bd* activity: in static and aeration conditions, MKs efficiently reduce iron and copper, while in respiration permissive conditions, this capacity is diminished following oxygen reduction by the cytochrome *bd* (Rezaiki *et al.*, 2004).

Haem toxicity management in *L. lactis*

While the importance of haem as a cofactor for respiration and numerous bacterial functions is well established (Reniere *et al.*, 2007b; Schultz *et al.*, 2010; Gruss *et al.*, 2012) the question remains as to what means are available to the cell for limiting haem toxicity due to iron bound to the protoporphyrin ring (forming ferriprotoporphyrin IX; FePPIX, haem). *L. lactis* encodes an orthologue of the Haem Regulated Transporter proteins, HrtB (permease) and HrtA (ATPase), which was first described in *Staphylococcus aureus* as involved in preventing haem toxicity, and whose inactivation results in haem hypersensitivity (Torres *et al.*, 2007; Pedersen *et al.*, 2008; Stauff *et al.*, 2008; Stauff and Skaar, 2009a). Our recent studies in *L. lactis* revealed that HrtBA functions directly as a haem-specific efflux pump, which we suggest is the case for HrtBA as present in other bacteria (Lechardeur *et al.*, 2012). However, unlike the situation in *S. aureus*, *hrtBA* regulation involves a transcriptional factor, HrtR, that controls the *hrtRBA* operon (Pedersen *et al.*, 2008; Lechardeur *et al.*, 2012). In the absence of haem, HrtR binds to the *hrtRBA* promoter, blocking transcription. High-affinity binding of HrtR to haem alleviates its interaction with the *hrtRBA* promoter, thus allowing transcription (Lechardeur *et al.*, 2012). HrtR functions as an intracellular haem sensor that plays a key role in haem intracellular homeostasis. Functional characterization of HrtBA in *L. lactis* expanded its suggested role and evolution of the *hrt* system beyond bacterial pathogens such as *S. aureus*, and gave insight into its direct function. In addition to efflux as a means of haem control, a recent study in *S. aureus* evoked a further role for MKs in haem toxicity (Wakeman *et al.*, 2012). In that study, mutants lacking enzymes of the MK synthesis pathway were found to have accrued resistance to environmental haem cytotoxicity. MK-mediated toxicity was attributed to oxidation of membrane-associated haem reservoirs (Wakeman *et al.*, 2012).

In this work, we investigated the link between haemin partitioning into membranes and HrtBA efflux activity, and how these two functions regulate *L. lactis* intracellular haem homeostasis. *L. lactis* is particularly suited for this study as haem levels are fully controlled by environmental pools. We observed that MKs and more generally reducing conditions in *L. lactis* correlate positively with the amounts of membrane-bound haem, while oxidative conditions have the opposite effect. HrtBA counteracts

MK-dependent membrane retention of excess haem. Finally, we investigated the possibility that haemin can reach the cytoplasm without the need for dedicated import systems for haem acquisition. Our conclusions lead to a new picture of haem intracellular acquisition and regulation in *L. lactis* in which HrtBA and the bacterial membrane have central roles.

Results

In this report, haem refers to iron protoporphyrin IX regardless of the iron redox state, whereas haemin refers to ferric iron protoporphyrin IX.

Added haemin is trapped in *L. lactis* membranes

Bacterial pellets of *L. lactis* grown with haemin (10 μ M) in a low-O₂ environment (static growth conditions) appeared strongly red (Fig. 1A, static). Colour intensity of pellets increased with increasing haemin concentrations (Fig. S1A). In contrast, bacteria remained white when grown in high-O₂ conditions (vigorous aeration) (Fig. 1A, aeration). Addition of haemin to *L. lactis* cultures in the presence of oxygen induces respiration metabolism (Dywat *et al.*, 2001; Pedersen *et al.*, 2012). The possibility that the contrasted colour phenotypes between static and aeration conditions could originate from respiratory chain activity was first investigated using two strains defective in key proteins of the respiratory chain; a subunit of the cytochrome *bd* oxidase and an enzyme of the MK biosynthesis pathway; respectively encoded by *cydA* and *menB* (Dywat *et al.*, 2001; Rezaiki *et al.*, 2004). As shown on Fig. 1A, the *cydA::ISS1* mutant behaved like the WT strain by accumulating haemin in static but not in aeration conditions. This observation suggested that activation of respiration metabolism was not responsible for the contrasted colour phenotypes of *L. lactis* in the presence of haemin. In contrast, the Δ *menB* mutant strain remained uncoloured compared to the WT strain (Fig 1A, Δ *menB*). Comparable results were also obtained when bacteria were grown in strict anaerobic fermentation conditions (data not shown). All together these first observations led us to conclude that *L. lactis* accumulates haemin by a mechanism driven by MK-mediated reduction and counterbalanced by oxygen availability. They suggested that low-oxygen conditions increase haemin (Fe³⁺PPIX) accumulation in *L. lactis* after reduction by MK. It is plausible that an oxidative aerobic environment prevents Fe³⁺PPIX reduction by interfering with MK reducing activity. To confirm this hypothesis, dithionite, a strong non-membrane-permeable reductant was added to the culture medium (Langner and Hui, 1993). As shown on Fig. 1B, dithionite restored haem sequestration in Δ *menB* mutants as a function of the concentration (0–5 mM). Addition of VitK2, a MK, had a similar effect. In

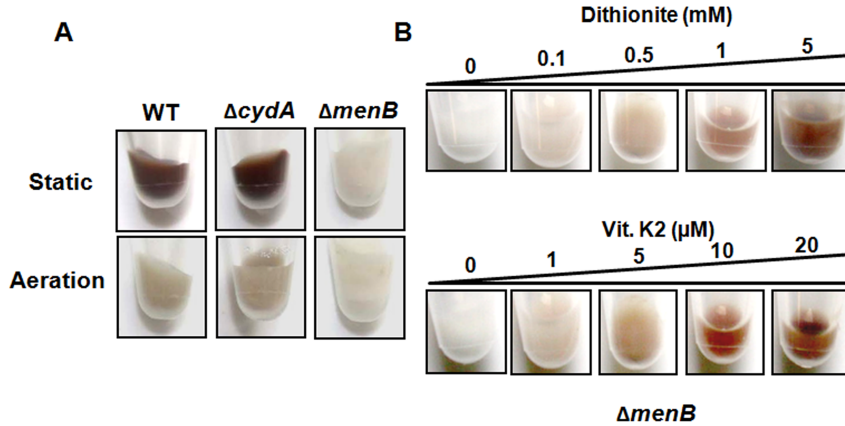


Fig. 1. *L. lactis* accumulates high levels of haem by an MK- and oxygen-dependent mechanism.

A. Colour phenotype of WT (MG1363), *cydA*::ISS1 (MG1363H1) and $\Delta menB$ (MG1363H2) strains in the presence of haemin. All *L. lactis* strains were grown to $OD_{600} = 0.5$ in static conditions prior to addition of 10 μM haemin to culture medium for an additional 1 h, in static or aeration conditions as indicated. Cells were pelleted by centrifugation.

B. Colour phenotype is restored in the $\Delta menB$ mutant (MG1363H2) by addition of dithionite or Vitamin K2 (a menaquinone). The $\Delta menB$ strain was grown in static conditions and incubated with 10 μM haemin together with the indicated concentrations of dithionite or Vitamin K2 for 1 h. Cells were pelleted by centrifugation. Result is representative of three independent experiments.

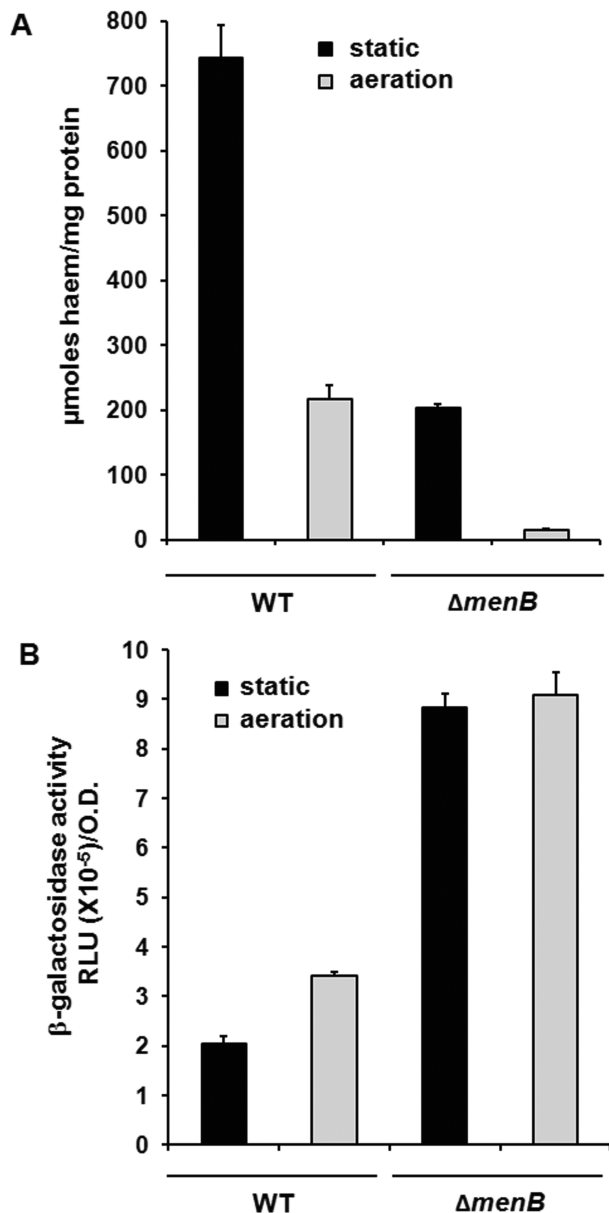
aeration conditions, addition of up to 2 mM dithionite did not lead to haem retention (data not shown), further suggesting that haemin is only poorly reduced in the presence of oxygen.

The above results indicate that Fe^{3+} PPIX is reduced to Fe^{2+} PPIX by membrane-located MKs, and is a strongly oxygen-sensitive event. Fast reversibility of retention when bacteria are transferred from static to aeration growth conditions further consolidates this conclusion (Fig. S1B). A role for MKs in haem redox status is in line with recent work of Wakeman *et al.* (2012) that gives evidence for MK-mediated reduction of haem retained in *S. aureus* membranes. Importantly, our work in *L. lactis* further reveals that the amount of cell-associated haem is also strongly dependent on environmental oxygen.

Both membrane and intracellular haem contents are MK-dependent in L. lactis

The subcellular localization of haem and its impact on haem intracellular homeostasis was analysed next. Accumulated haem remained associated with the bacteria following treatment with lysozyme, suggesting that accumulated haem was not associated with the cell wall (data not shown). Membrane and intracellular haem pools were quantified in WT and $\Delta menB$ bacteria exposed to haemin. Haem quantification in isolated membranes was assessed using the pyridine haemochrome assay (Fig. 2A). When grown in static conditions, membranes from WT strains contained about four times more haem than membranes from $\Delta menB$ strains (Fig. 2A), showing that haemin associates readily with membranes when MKs are present.

Enrichment of the culture medium with oxygen through aeration almost completely abolished the presence of haem in both WT and $\Delta menB$ membranes (Fig. 2A). The intracellular haem content was evaluated under the same conditions. Direct quantification of haem in the cytoplasmic fraction was not performed, as our results suggested that high concentrations of added haem might contaminate the cytoplasmic fraction during preparation. We therefore made use of the HrtR intracellular haem sensor to quantify haem intracellular concentrations, as shown previously (Lechardeur *et al.*, 2012). Intracellular haem concentrations were followed using $P_{hrt}hrtR-lac$ as reporter established in the WT and $\Delta menB$ strains (Fig. 2B). $P_{hrt}hrtR-lac$ is virtually off in the absence of haemin validating the haemin requirement for β -gal induction (Lechardeur *et al.*, 2012). In static growth conditions, β -gal activity was about fourfold higher in $\Delta menB$ strains compared to WT exposed to 5 μM haemin, a concentration that did not saturate the HrtR mediated response system (Lechardeur *et al.*, 2012). These results suggested that the defect in MK synthesis led to a significant increase of intracellular haem (Fig. 2B). Western blot analysis using HrtR-directed antibodies further confirmed the higher intracellular haem balance in the $\Delta menB$ mutant compared to the WT strain in static conditions (Fig. S2A). Unlike the case for membrane haem, amounts of intracellular haem were higher in the WT strain grown in aeration compared to static growth conditions (Fig. 2B and Fig. S2B). We noted that haemin toxicity was minimal at the tested concentration (5 μM) and conditions, although the $\Delta menB$ strain was less sensitive to haemin than the WT at higher concentrations (Fig. S3). These results show that exogenously added haem parti-



tions between the membrane and intracellular compartments, and that MKs impact its differential localization.

HrtBA mediated efflux prevents haem sequestration in the L. lactis membrane

A role for HrtBA in reducing haem toxicity was first reported in *S. aureus* and shown in several bacteria (Reniere *et al.*, 2007a; Torres *et al.*, 2007; Stauff and Skaar, 2009b; Bibb and Schmitt, 2010; Burgos and Schmitt, 2012; Juarez-Verdayes *et al.*, 2012; Lechardeur *et al.*, 2012). Our studies in *L. lactis* further implicated HrtBA as responsible for efflux of haem intracellular pools (Lechardeur *et al.*, 2012). However, the recent work of Skaar and col-

Fig. 2. Both membrane and intracellular haem contents are modified upon oxygen availability and menaquinone activity. A. Haem is sequestered in membranes of *L. lactis* grown in static conditions. WT (MG1363) and $\Delta menB$ (MG1363H2) strains were grown in static conditions to $OD_{600} = 0.5$ prior addition of $3 \mu M$ haemin in the indicated growth conditions. Control bacteria were grown in the same conditions without haemin (not shown). Bacteria were pelleted and membranes isolated (see *Experimental procedures*). Membrane fractions were solubilized in 50 mM Tris-Cl, pH 7.5 containing 150 mM NaCl and 0.2% Triton X-100. Haem content was determined by the pyridine haemochrome assay and normalized to protein concentration (see *Experimental procedures*). Results represent the mean \pm standard deviation from triplicate samples. Result is representative of three independent experiments.

B. Induction of the HrtR intracellular haem sensor by haemin in WT (MG1363) and $\Delta menB$ (MG1363H2) strains grown in static and aeration growth conditions. The strains carrying $P_{hrt}hrtR-lac$ were grown to $OD_{600} = 0.5$. β -gal activity was determined after 1 h incubation with $3 \mu M$ haemin as in (A) and quantified by luminescence (*Experimental procedures*). Results represent the mean \pm standard deviation from triplicate samples. Values of luminescence obtained with no added haem are subtracted. Result is representative of three independent experiments.

leagues (Wakeman *et al.*, 2012) linked MK-dependent haem sequestration in membranes to haem toxicity, which was alleviated in *S. aureus* by HrtBA activity, but not to haem quantity. We asked whether HrtBA also manages membrane haem levels, and exploited the fact that haem pools in *L. lactis* are fully exogenously controlled. To investigate this, we evaluated membrane haem sequestration in *L. lactis* strains either lacking the entire *hrtRBA* operon or overexpressing the HrtBA transporter (Lechardeur *et al.*, 2012). In static growth conditions, the amount of haem sequestered in WT *L. lactis* was dependent on the expression of HrtBA (Fig. 3A). Overproducing the HrtBA transporter [$\Delta hrtRBA(phrtBA)$] almost completely abrogated haem accumulation, while $\Delta hrtRBA$ mutant bacteria were more intensely coloured than the WT strain (Fig. 3A). Haem quantification in isolated membranes from WT, $\Delta hrtRBA(phrtBA)$ and $\Delta hrtRBA(pCtrl)$ bacteria exposed to $3 \mu M$ haemin confirmed that HrtBA overproduction prevented accumulation of haem in membranes (Fig. 3B). These data are consistent with the conclusion that HrtBA regulates the amounts of cellular haem both at the intracellular and at the membrane levels.

HrtBA competes with quinone haemin reducing activity to control L. lactis haem content

The finding that both HrtBA and MKs impact haem accumulation in cytoplasmic and membrane compartments might imply that haemin reduction, e.g. by MKs, is a prerequisite for HrtBA-mediated efflux. To study the impact of membrane haem retention on HrtBA function, we relied on the colour phenotype of bacteria. While haem sequestration in membranes was lower in aeration conditions (WT,

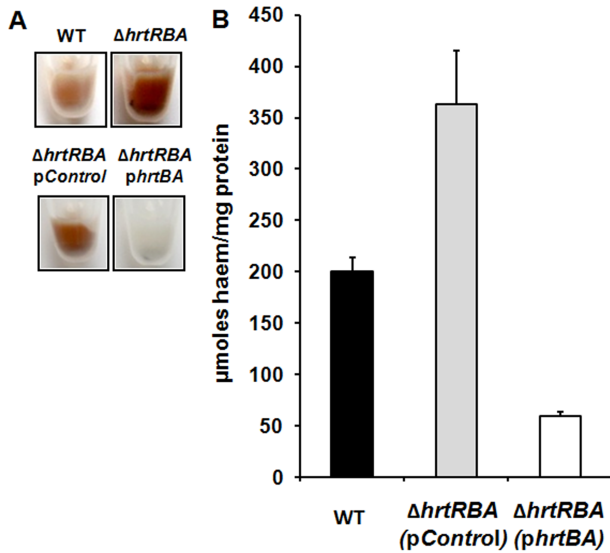


Fig. 3. The HrtBA transporter controls membrane haem content via efflux in *L. lactis*.

A. HrtBA participates in the colour phenotype of *L. lactis* upon haemin exposure. WT (MG1363), $\Delta hrtRBA$ (MG1363H3), $\Delta hrtRBA(pControl)$ or $\Delta hrtRBA(phrtBA)$ were grown in static conditions to $OD_{600} = 0.5$ prior addition of $3 \mu M$ haemin to the culture for 1 h. Cells were pelleted by centrifugation. Result is representative of three independent experiments.

B. HrtBA overproduction prevents membrane accumulation of haemin. Pyridine assay was performed on membranes isolated from bacteria exposed to $3 \mu M$ haemin as in (A). Haem content was normalized to the protein concentration (see *Experimental procedures*). Results represent the mean \pm standard deviation from triplicate samples. Result is the mean of three independent experiments.

aeration) compared to static conditions, the $\Delta hrtRBA$ mutant exhibited significantly stronger staining in both growth conditions (Fig. 4A). When the same assay was performed in a $\Delta menB$ background, i.e. comparing $\Delta menB$ and $\Delta menB \Delta hrtRBA$, the $\Delta menB \Delta hrtRBA$ strain was slightly more coloured than the $\Delta menB$ strain. In other words, even if membrane sequestration of haem is low in oxidizing conditions (Fig. 4A), or in the $\Delta menB$ mutant (Fig. 4B), HrtBA is needed to drive out residual haem. These data support the idea that haemin reduction by MKs is not a prerequisite to HrtBA control of haem homeostasis at the membrane level.

We next investigated the impact of inhibiting haemin reduction on the capacity of HrtBA to regulate haem intracellular pools. In Fig. 2B, we showed that $P_{hrt}hrtR-lac$ induction was optimal in conditions where haemin is not reduced by MKs (i.e. in aeration conditions and in a $\Delta menB$ mutant). Increased intracellular haem could result from inhibition of haem efflux by HrtBA, but also from greater haem uptake. To discriminate between these possibilities, the amount of intracellular haem was tested independently of HrtBA efflux activity by comparing $\Delta menB \Delta hrtRBA$ and

the $\Delta hrtRBA$ mutant (Fig. 4C). In static growth conditions, $\Delta menB$ deletion led to a significant increase of intracellular haem as seen by β -gal activity from $pP_{hrt}hrtR-lac$ (Fig. 4C; note that $10 \mu M$ haem is not toxic in the $\Delta menB$ strain) This

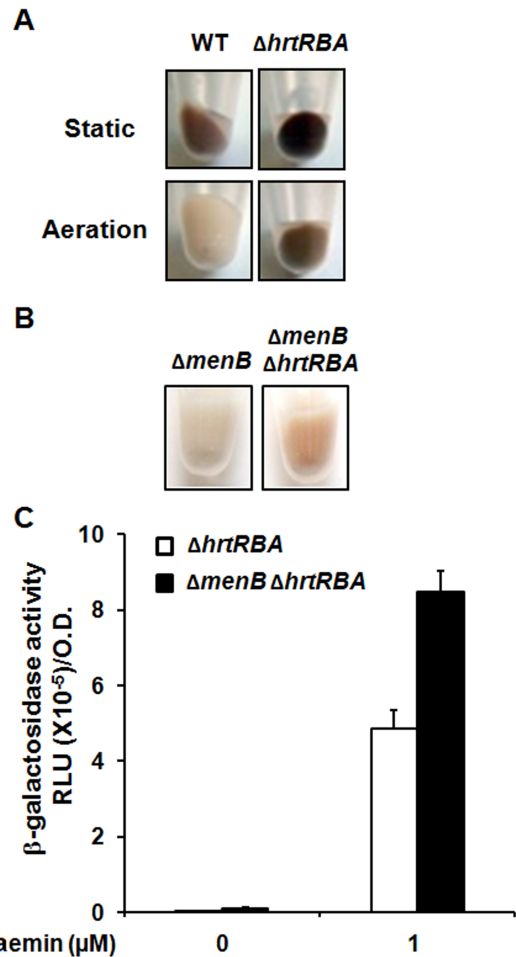


Fig. 4. HrtBA is functional in conditions when haem does not accumulate in membranes.

A. HrtBA participates in the colour phenotype of *L. lactis* upon haemin exposure in both static and aeration growth conditions. WT (MG1363), $\Delta hrtRBA$ (MG1363H3) *L. lactis* cultures were grown in static conditions to $OD_{600} = 0.5$ prior addition of $10 \mu M$ haemin to the culture grown for an additional 1 h in static followed by 5 min in aeration conditions as indicated. Cells were pelleted by centrifugation. Result is representative of three independent experiments.

B. Comparative colour phenotype of $\Delta menB$ (MG1363H2) and $\Delta menB \Delta hrtRBA$ (MG1363H4) *L. lactis*. Mutant strains were grown in static conditions to $OD_{600} = 0.5$. $10 \mu M$ haemin was added to the culture for 1 h and bacteria were harvested by centrifugation. Result is representative of three independent experiments.

C. Intracellular haem content of $\Delta menB$ (MG1363H2) and $\Delta menB \Delta hrtRBA$ (MG1363H4) strains. Strains carrying $P_{hrt}hrtR-lac$ were grown to $OD_{600} = 0.5$. β -gal activity was induced for 1 h in the presence of the indicated concentrations of haemin and quantified by luminescence (*Experimental procedures*). Results represent the mean \pm standard deviation from triplicate samples and are representative of three independent experiments.

result supports the notion that the observed increase of intracellular haem in $\Delta menB$ or in the WT strain grown in aeration is due to greater haem uptake, independently of HrtBA efflux activity. This result was further confirmed by showing that overexpressing HrtBA in the $\Delta menB \Delta hrtRBA$ strain (Fig. S4) led to a considerable decrease of intracellular haem as seen by the expression of $pP_{hrt}hrtR-lac$, β -gal activity, similarly to what was observed in the WT strain (Lechardeur *et al.*, 2012). Altogether, these results suggest that membrane and intracellular haem homeostasis in *L. lactis* is the result of two competing but independent mechanisms with opposite effects, i.e. sequestration favoured by MKs and export by HrtBA.

Haem acquisition is not dependent on surface ABC transporters in *L. lactis*

The results described above point out that haem acquisition in *L. lactis* as tested by the HrtR-based haem intracellular haem sensor depends on the combined activities of both environmental or membrane reduction capacity, and HrtBA-mediated haem efflux. These results led us to ask whether haemin acquisition in *L. lactis* could occur by membrane diffusion. While the lipophilic character of haemin could be compatible with diffusion, haemin import in Gram-positive bacteria has been shown to rely on ABC-transporters associated with membrane-anchored lipoproteins (for review, Gruss *et al.*, 2012). To discriminate between these two possibilities in *L. lactis*, we relied first on sodium orthovanadate, a potent inhibitor of ATPases that blocks ABC-transporter functions implicated in most import systems (Garvey and Piddock, 2008). The impact of ATPase inhibition on haem acquisition was examined in $\Delta hrtRBA$ strains transformed with the $pP_{hrt}hrtR-lac$ reporter. Cells pre-treated with 2 mM orthovanadate for 0.5 h followed by 1 h induction with 1 μ M haemin (non-toxic in the $\Delta hrtRBA$ strain) exhibited β -gal activity that was only slightly diminished compared to control bacteria (Fig. 5). HrtBA is an ATP-dependent transporter and presumably orthovanadate-sensitive. Under the same conditions, haemin intracellular content in the $\Delta hrtRBA(phrtBA)$ strain, used as a control of orthovanadate treatment, was over fivefold increased, thus validating the effect of orthovanadate in inhibiting the HrtA ATPase (Fig. S5). These results suggested that in the experimental μ M haem concentrations used in the present study, haem intracellular import is mainly independent of the function of ABC transporters. These data are compatible with the notion that haem import is not mediated by lipoproteins associated with ABC transporters. These observations are consistent with our proposal that haem import in *L. lactis* occurs by membrane diffusion and is modulated by the combined actions of reducing activity via MKs and HrtBA-mediated efflux.

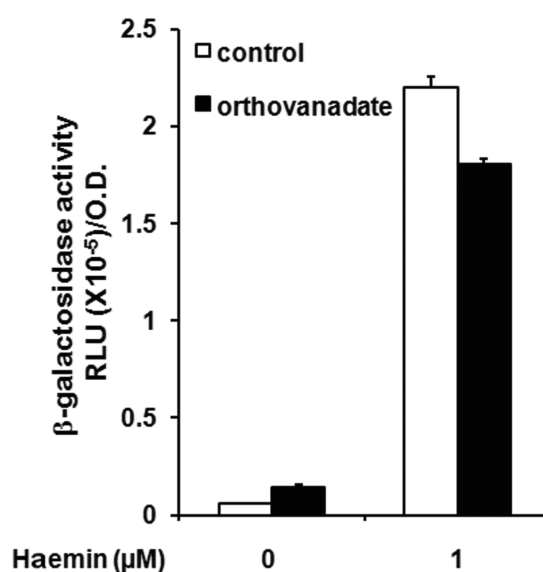


Fig. 5. Surface ABC transporters are not involved in haemin transport to the cytoplasm. Inhibition of ATPases with orthovanadate has a minimal impact on intracellular haem homeostasis. The $\Delta hrtRBA$ (MG1363H3) strain carrying $P_{hrt}hrtR-lac$ was grown to $OD_{600} = 0.5$ and split in two. Cultures were pre-incubated for 0.5 h without or with 2 mM orthovanadate, followed when indicated by the addition of 1 μ M haemin for 1 h. Luminescence was determined as described (*Experimental procedures*). Results represent the mean \pm standard deviation from triplicate samples and are representative of three independent experiments.

Two conserved tyrosine residues localized in the large extracellular domain of HrtB are essential for haem efflux

In an effort to decipher the mechanism of haem efflux from membranes by HrtB, we looked for amino acids (AAs) in HrtB that might be implicated in haem binding, e.g. as reported for the haem transporter CcsBA in *Escherichia coli* (Frawley and Kranz, 2009). HrtB is predicted to comprise four transmembrane segments and one large extra-cytoplasmic loop (ECL) comprising AAs 41 to 237 (by TopPred 0.01, mobyle.Pasteur.fr; Fig. 6A). Alignment of HrtB sequences from several strains revealed two conserved tyrosines that might serve as haem axial ligands localized at positions 168 and 231 on the predicted ECL (Fig. 6A and Fig. S6). To investigate a possible implication of these two tyrosines in haem efflux by HrtB, both were substituted with leucines either separately or together by overlap mutagenesis using a plasmid expressing *hrtBA* (see *Experimental procedures*), and generating mutant proteins Y168L and Y231L as well as a double mutant Y168L, Y231L (Tables S1 and S2). WT and mutant HrtB together with HrtA were overexpressed in the $\Delta hrtRBA$ mutant background. To verify that the mutations did not impact HrtB expression or its insertion in the membrane, an

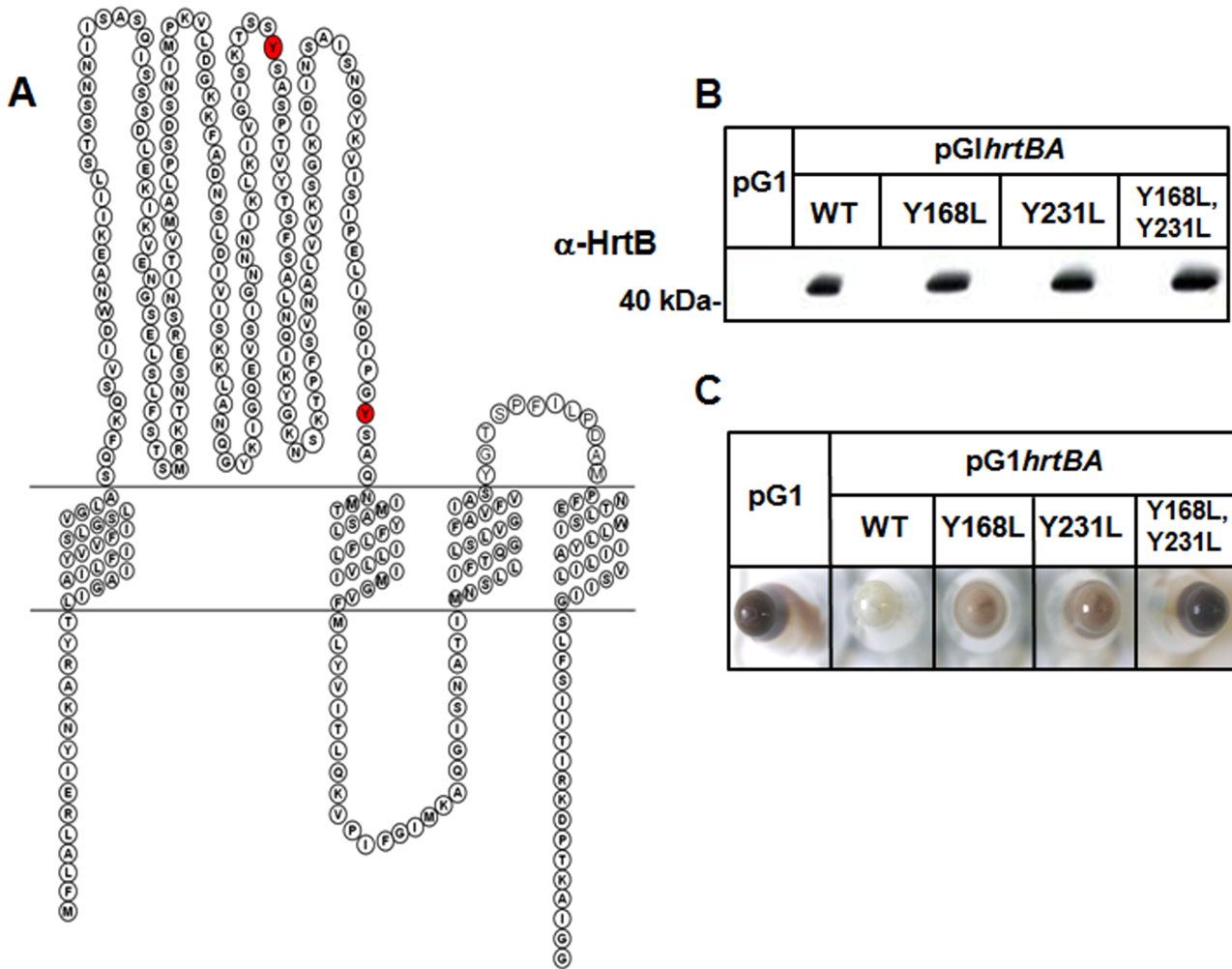


Fig. 6. Two conserved tyrosines are required for HrtB function.

A. Predicted membrane topology of HrtB. HrtB hypothetical insertion in the membrane was drawn from its predicted membrane topology and is characterized by four transmembrane segments and a large extracytoplasmic domain comprising the [41–237] domain. Two conserved tyrosines are highlighted in red.

B. WT HrtB, and Y168L, Y231L and YY variants are expressed similarly in *L. lactis* membranes. An antibody raised against the ECL domain of HrtB (see *Experimental procedures*) was used on Western blots of membranes isolated from bacteria overexpressing the HrtBA WT or with the HrtB variants overexpressing the WT HrtBA transporter or single Y168L and Y231L as well as double Y168L, Y231L (YY) HrtB mutations.

C. Mutations of two conserved tyrosines (Y) of the ECL domain inhibit haem efflux by HrtB. Colour phenotype of Δ hrtRBA strains incubated with 10 μ M haemin as in Fig. 1, overproducing the WT or mutant HrtBA transporter (as in B).

antibody recognizing the predicted ECL (Fig. 6A) was obtained with purified His-HrtB-ECL (data not shown). Both modified and wild-type HrtBA proteins displayed similar HrtB expression levels in membranes in Western blots (Fig. 6B), and on the cell surface by immunofluorescence microscopy of fixed non-permeabilized cells (Fig. S7). These results confirmed that the predicted ECL comprising AAs 37 to 237 was extracellular and that the mutation did not detectably modify HrtB localization. Similar results were obtained with the single mutants (data not shown). When incubated with 5 μ M haemin in static conditions, cells overexpressing WT HrtBA showed essentially no haem accumulation when compared to the control

Δ hrtRBA strain (Fig. 6C). In contrast, bacteria overproducing either HrtB mutant-HrtA couple accumulated significantly more haemin as seen by the bacterial pellet phenotype (Fig. 6C). In the same conditions, bacteria overexpressing the doubly mutated HrtB and HrtA displayed a Δ hrtRBA minus phenotype. The colour phenotype observed with the tyrosine mutants probably arose from HrtB dysfunction linked to haem efflux. While the two conserved tyrosines are potential haem ligands, we cannot rule out that their mutations alter overall conformation of HrtB-ECL. Additional studies will thus be needed to validate that haem binding to ECL participates in the haem efflux mechanism. These data highlight the importance of the large ECL

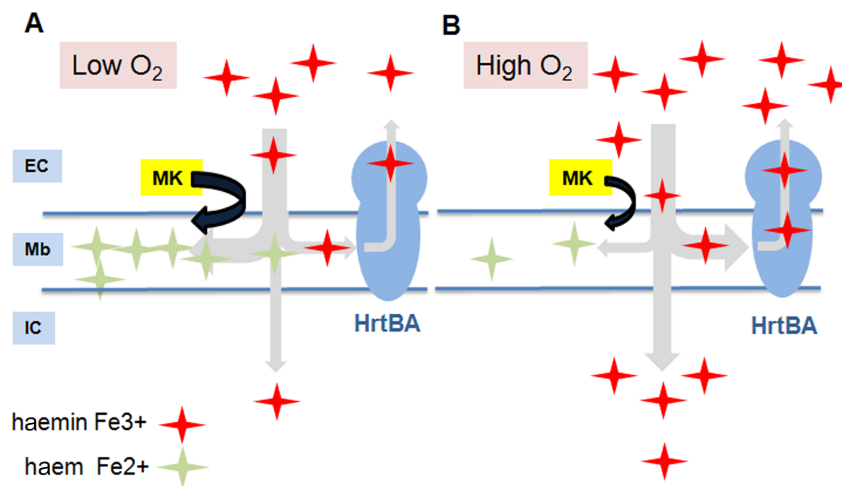


Fig. 7. Schematic representation of the control of haem homeostasis by menaquinones and HrtBA in *L. lactis*. Extracellular (EC) haemin (red star) enters the intracellular (IC) space through the membrane (Mb) compartment. Along this path, haemin is either sequestered in the membrane following its reduction to haem (green star) by MK (or other) reductase activity, or intercepted and effluxed back to the medium. Reduction of extracellular haemin by MKs is more frequent in low oxygen (A), than in a high-oxygen environment (B). The degree of haem immobilization in membranes is also counterbalanced by HrtBA-mediated haemin efflux that further limits MK reduction. Intracellular homeostasis of acquired haemin thus depends on both redox via MK and oxygen, and HrtBA activities in the *L. lactis* membrane.

domain in the efflux mechanism of HrtB and are compatible with our finding that HrtBA has a specific role in effluxing membrane haem.

Discussion

Acquired haemin accumulates in membranes by a mechanism involving menaquinone and oxygen

In this work, we observed that haem accumulates in *L. lactis* membranes by a mechanism that depends on reducing activity, notably due to endogenous membrane-localized menaquinones (MKs). An oxidative environment, provided by oxygen, prevents and reverses haemin reduction and thus limits haem accumulation in membranes. The implication of MKs in the reduction of metals present in bacterial environment (Cu, Fe) has been reported (Duwat *et al.*, 2001; Rezaiki *et al.*, 2004; Richard-Fogal *et al.*, 2009; Abicht *et al.*, 2013). We extend the previous findings to suggest that MKs may also reduce Fe in the haem molecule. However we cannot exclude the implication of yet unidentified MK-dependent haemin reductase(s). The question of why reduced haemin accumulates in membranes remains to be investigated. However, lack of saturation and fast reversibility upon oxygen addition favour the idea that reduced haemin partitions into the membrane lipid bilayer because of its lipophilicity rather than by binding to protein targets. Our results provide new information that modifies previous conclusions of Wakeman *et al.* (2012) on the role of MKs on haem toxicity in aerobically grown *S. aureus*. Their data suggested that membrane-associated haem and quinone molecules form a redox cycle that continuously generates semi-quinones and reduced haem to produce cytotoxic superoxides (Wakeman *et al.*, 2012). Our results in *L. lactis* support the conclusion that in lower oxygen conditions, while MKs reduce larger pools of haemin than in aerated conditions,

the bacteria can probably tolerate larger membrane haem pools because of their lower reactivity.

HrtBA prevents haem accumulation in membranes by direct efflux

Recently, we established that HrtBA was responsible for intracellular haem homeostasis by direct haem efflux in *L. lactis* (Lechardeur *et al.*, 2012). Here we show that HrtBA also controls membrane-localized haem pools, thus providing a mechanistic explanation for the role of HrtBA in regulating haem toxicity (Torres *et al.*, 2007; Anzaldi and Skaar, 2010; Lechardeur *et al.*, 2012). Our results are consistent with the hypothesis that HrtBA effluxes haem from the membrane compartment before it reaches the intracellular space. We propose a model in which membrane MK reducing activity favours haem retention while HrtBA activity prevents this effect by transporting the toxic molecule back to the medium from the membrane (Fig. 7). In view of these results, the HrtBA efflux mechanism could be similar to that of efflux transporters of non-polar molecules known to partition into the lipid bilayer where they accumulate to high concentrations (Sharom, 2008). Similar mechanisms are considered for efflux pumps that confer multidrug resistance to antibiotics and chemotherapeutic drugs, both in prokaryotes and in eukaryotes (Lage, 2003). In the latter examples, MDR family efflux pumps likely function as 'hydrophobic vacuum cleaners' or 'flippases', which expel hydrophobic molecules into the extracellular medium from the membrane, thus preventing them from reaching their intracellular targets (Higgins and Gottesman, 1992; Sharom, 1997; 2008; Eckford and Sharom, 2009). The overall conformation of HrtB, including a large extracellular domain with potential substrate specificity, is also reminiscent of transporters involved in efflux of hydrophobic molecules (Fig. 6) (Murakami *et al.*, 2002; Sharom, 2008; Frawley and Kranz, 2009; Xu *et al.*, 2009;

Desuzinges-Mandon *et al.*, 2010). Our results are thus consistent with the hypothesis that HrtBA drives out membrane haem, and further indicate that HrtRBA is the sole dedicated regulator of haem homeostasis in *L. lactis*.

Haem acquisition in *L. lactis*

Altogether our findings that MKs and HrtBA control haem intracellular homeostasis in *L. lactis* favour the notion that haem entry is not rate-limiting. While significant progress has been made in identifying and understanding the mechanisms responsible for haem transport in a few Gram-positive bacteria including *S. aureus* (Mazmanian *et al.*, 2003; Skaar and Schneewind, 2004; Maresso *et al.*, 2008; Gruss *et al.*, 2012), how haem is internalized in most bacteria remains poorly described. We give strong evidence that, in the conditions tested, haem crosses the *L. lactis* membrane by a mechanism that does not imply surface transporters. Diffusion of several lipid-soluble compounds across membranes is documented for example, for small organic molecules ligands of TetR family regulators (Ramos *et al.*, 2005). In Gram-positive bacteria, the accepted uptake model for soluble molecules relies on membrane-anchored lipoprotein ABC transporters that bind and transfer specific ligands to a transmembrane permease. The transported molecules thereby circumvent contact with the hydrophobic interior of the lipid bilayer (Sharom, 1997). According to our data, haem entry into the intracellular space of *L. lactis* is optimal when its retention in membranes is minimal (i.e. when haemin is *not* reduced) (Fig. 2). This would suggest that Fe³⁺PPIX freely diffuses across the membrane while Fe²⁺PPIX accumulates within it (Fig. 7). The extent of haem partitioning into the membrane could therefore dictate the rate of entry of the non-reduced haemin. Further biophysical studies will be needed to determine the exact mechanism of haem interaction with the membrane.

Based on our data, we propose that in the μM range, free haem entry in *L. lactis* is dictated by redox activities associated with MKs and oxygen, and HrtBA-mediated efflux of membrane-localized haem. It is conceivable that in conditions where haem concentrations are low, expression of membrane transporters combined with high-affinity haemophores are necessary for haem acquisition in *L. lactis*.

Experimental procedures

Bacterial strains and growth conditions

The strains and plasmids used in this work are listed in Table S1. Experiments in *L. lactis* were all performed using strain MG1363 and derivatives. Overnight pre-cultures were grown at 30°C in M17 medium supplemented with 1% glucose. They

were then diluted and grown in M17 with 0.2% glucose. Aerated cultures were obtained by growing strains in Erlenmeyer flasks with a 1:20 medium : volume ratio in a rotary shaker (200 r.p.m.). Cultures grown in static conditions were considered as microaerobic. When needed, antibiotics were used for *L. lactis* at 5 $\mu\text{g ml}^{-1}$ erythromycin and 5 $\mu\text{g ml}^{-1}$ tetracycline; for *E. coli* at 50 $\mu\text{g ml}^{-1}$ kanamycin. Liquid cultures were generally grown to OD₆₀₀ = 0.5 and then incubated for 1 h with the test porphyrin at the indicated concentration. Oligonucleotides used for plasmid constructions are listed in Table S2. Haemin (FePPIX) (Frontier Scientific) stock solution (10 mM) was prepared in 50 mM NaOH. All other chemicals were from Sigma Aldrich.

β -Galactosidase assay

β -Galactosidase (β -gal) activity was assayed on bacteria carrying the pP_{hrtB}hrtR-lac plasmid as described (Lechardeur *et al.*, 2012). β -gal was quantified by luminescence in an Infinite M200 spectroluminometer (Tecan, Austria) using the β -Glo assay system as recommended by manufacturer (Promega).

Anti-HrtB antibody

An anti-HrtB antibody targeted to the extracellular loop (ECL) corresponding to AAs [41–337], HrtB-ECL, was prepared with His-tagged HrtB-ECL as antigen, purified from bacterial lysates on a nickel affinity resin (Sigma Aldrich) as described (Lechardeur *et al.*, 2012). The resulting purified His-HrtB-ECL was used for rabbit antibody production (Covalab, France). Antiserum specificity was determined by Western blots using known amounts of purified His-HrtB-ECL proteins and bacterial lysates expressing HrtB.

Bacterial lysate preparation

Bacteria were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). Resuspended cells were then pelleted at 3500 *g* for 10 min, resuspended in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, containing 0.2% Triton X-100 (lysis buffer), and disrupted with glass beads (Fastprep, MP Biomedicals). Cell debris was removed by centrifugation at 18 000 *g* for 15 min at 4°C.

Membrane isolation and pyridine assay

Bacteria were pelleted by centrifugation at 3500 *g* for 10 min and resuspended in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and were lysed with glass beads (Fastprep, MP Biomedicals). Cell debris was removed by centrifugation at 18 000 *g* at 4°C for 15 min and cell membranes were sedimented at 100 000 *g* for 45 min at 4°C. Membrane pellets were resuspended in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2% Triton X-100. Proteins were quantified by the Lowry Method (Bio-Rad) and equivalent amounts (in a volume of 65 μl) were mixed with 10 μl NaOH 1 M, 20 μl pyridine and 5 μl sodium dithionite 100 mM or 5 μl of potassium ferricyanide 20 mM. Absorption spectra of the samples were recorded from 500 nm and

600 nm in an Infinite M200 spectroluminometer (Tecan). Dithionite-reduced minus ferricyanide-oxidized spectra of pyridine haemochromes from membrane-enriched fractions were used to determine the amount of haem *b* by following the absorbance change at 557 nm using a difference extinction coefficient of 28.15 mM⁻¹ cm⁻¹ (Berry and Trumpower, 1987).

Acknowledgements

We thank Cécile Wandersman (Institut Pasteur, Paris), Philippe Delepelaire (Institut de Biologie Physico-Chimique, Paris), and Elise Borezée (INRA) for helpful discussions.

This work was supported by the HemeStockExchange project by the French 'Agence Nationale de la Recherche' ANR-12-BSV3-022-01 and by funding from the French National Research Agency ANR-11-IDEX-0003-02; 'ALIAS' project.

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