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Identification of the Essential *Brucella melitensis* Porin Omp2b as a Suppressor of Bax-Induced Cell Death in Yeast in a Genome-Wide Screening

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**Abstract**

**Background:** Inhibition of apoptosis is one of the mechanisms selected by numerous intracellular pathogenic bacteria to control their host cell. *Brucella*, which are the causative agent of a worldwide zoonosis, prevent apoptosis of infected cells, probably to support survival of their replication niche.

**Methodology/Principal Findings:** In order to identify *Brucella melitensis* anti-apoptotic effector candidates, we performed a genome-wide functional screening in yeast. The *B. melitensis* ORFeome was screened to identify inhibitors of Bax-induced cell death in *S. cerevisiae*. *B. melitensis* porin Omp2b, here shown to be essential, prevents Bax lethal effect in yeast, unlike its close paralog Omp2a. Our results based on Omp2b size variants characterization suggest that signal peptide processing is required for Omp2b effect in yeast.

**Conclusion/Significance:** We report here the first application to a bacterial genome-wide library of coding sequences of this “yeast-rescue” screening strategy, previously used to highlight several new apoptosis regulators. Our work provides *B. melitensis* proteins that are candidates for an anti-apoptotic function, and can be tested in mammalian cells in the future. Hypotheses on possible molecular mechanisms of Bax inhibition by the *B. melitensis* porin Omp2b are discussed.

**Introduction**

During the complex evolution of intracellular pathogenic bacteria with their host, mechanisms have been selected that allow bacteria to modulate host cell functions to their advantage. Examples of hijacking of the host cell cytoskeleton [1], signaling [2] and vesicular trafficking [3] by bacterial pathogens are numerous. Pirated processes include apoptosis, a form of programmed cell death that can serve as a defense mechanism to avoid pathogen propagation [4]. Inhibition of apoptosis, which has been reported for a variety of intracellular bacteria, probably supports survival of their replication niche [5].

Monocytes or macrophages infected by the facultative intracellular pathogenic bacteria of the *Brucella* genus, which are responsible for a worldwide zoonosis [6], are protected from apoptosis [7]. Furthermore, modulation of the expression of genes encoding apoptotic proteins in infected cells has been described [7,8]. The gene encoding an anti-apoptotic protein of the Bcl-2 family, A1/Bcl1, is overexpressed in *Brucella*-infected monocytes compared with non-infected cells [7]. However, the bacterial molecular mechanisms leading to apoptosis inhibition, probably involving production of effector protein(s), are still unknown.

Interestingly, the genetically tractable and easy to handle yeast *Saccharomyces cerevisiae* is known to be an attractive model for the functional study of proteins involved in apoptosis, such as the Bcl-2 family members [9]. The budding yeast lacks homologs of the mammalian core apoptotic proteins, providing a simplified model for individual characterization of apoptosis regulators without the bias due to the rest of the network. Several proteins involved in apoptosis have an effect in yeast that is relevant to their physiological function [9]. For instance, the ectopic production of the mammalian pro-apoptotic Bax in yeast induces a form of cell death, which requires Bax insertion into the outer membrane of mitochondria (OMM). In mammalian cells, Bax translocation to mitochondria occurs upon pro-apoptotic signaling and leads to mitochondrial outer membrane permeabilization (MOMP) [10], a required event for subsequent apoptosis. Pleiotropic effects are observed upon Bax production in yeast, including several features shared with mammalian apoptosis such as MOMP, cytochrome c release and the maintenance of plasma membrane integrity [11].

These characteristics, in addition to the fact that Bax-induced cell death in yeast can be reverted by known anti-apoptotic proteins, such as Bcl-2 and Bcl-XL [12], strongly support a common ancestral pathway to controlled cell death. Hence, *S. cerevisiae* has
been used as a tool for the identification of new putative apoptosis inhibitors. A screening strategy to identify inhibitors of Bax-induced cell death in yeast [13] has been applied previously to identify new human anti-apoptotic proteins [14,15,16] and plant inhibitors of cell death [17,18]. In addition, assaying of Bax-induced cell death inhibition in yeast has contributed to the characterization of human [16] and plant [19] proteins known to be involved in inhibition of apoptotic or hypersensitive response (HR) programmed cell deaths, respectively, as well as bacterial proteins playing a role in host HR cell death inhibition [20] or described as secreted anti-apoptotic effector [21].

Several bacterial effectors that target eukaryotic signaling pathways have been highlighted using diverse functional screening approaches in yeast [22]. The availability of the Brucella melitensis ORFeome prompted us to perform a genome-wide functional yeast-based screening to identify B. melitensis anti-apoptotic effector candidates. Here we report the results of a screening for Bax suppressors in yeast, applied for the first time to a bacterial genome-wide library of coding sequences.

Results and Discussion

In order to identify Brucella melitensis proteins that inhibit Bax-induced cell death in yeast, we took advantage of the B. melitensis ORFeome [23], which is a standardized library of predicted coding sequences (ORFs), to construct a yeast library in which each clone contains murine bax and one of the ~3,200 B. melitensis ORFs. bax and the B. melitensis ORFs expression are controlled by the galactose-inducible, glucose-repressible pGAL10 and pGAL1 promoters, respectively. This “bax + ORFeome” library was screened for colonies with a plasmid-born growth phenotype under bax and ORFs expression condition (see Text S1 in the Supporting information for library construction and further screening details). 116 different ORFs were identified from the 136 colonies obtained at this step of the screen. In order to get rid of false positives that are expected to be selected in this type of large-scale screen of a genome-wide pool of ORFs, the next step of the screening procedure consisted in individually testing all candidates in conventional growth assays [13]. Thus, the 116 pYES-DEST52 ORFs were individually recloned from the original entry vectors of the ORFeome before transformation of a yeast strain containing bax (QX95001, see Table 1 for all strains used in this study). Each of the 116 strains was submitted to growth assays (streaks and drops of serial dilutions on plate) under induction or repression conditions of bax and ORFs expression. The positive control of growth was GL0001 yeast strain containing bax and bclXL. Indeed, the anti-apoptotic Bcl-XL protein has been shown to inhibit Bax-induced cell death in yeast [12] as in mammalian cells by preventing Bax insertion into mitochondria and its acquisition of a final conformation able to induce MOMP [24]. The negative control was the GL0016 yeast strain containing bax and the empty pYES-DEST52 destination vector. Out of the

Table 1. Yeast and bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Strain</th>
<th>Use and relevant features</th>
<th>Medium</th>
<th>Reference and sources</th>
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| DH10B      | E. coli F- mcrA (Δmrr-hsdRMS-mczB) phl80 lacZΔM15 lacIVΔ4 recA1 endA1 araΔA19 bαara leu7697 galU galK lambda Δrps (Str+)
|            |                                  | cloning strain                                   | LB              | Gibco BRL             |
| S17-1      | E. coli recA thi pro hsdR [res- mod+]
|            |                                  | plasmid mobilization in B. melitensis           | LB              | [41]                  |
| BF264-15 Dau | S. cerevisiae (MATAlpha ade1 his2 leu2-3, 112 thr1-1 ura3) | yeast strain used in this study for transformation with plasmids harboring LEU2 or URA3 | Sc              | [42]; kindly provided by M. Grant, Wye College, UK |
| GL0001     | QX95001 + pYES-DEST52-bclXL      | pGAL10-bax + pGAL1-bclXL-V5-His6                | Sc-Leu-Ura      | This study            |
| GL0002     | BF264-15 Dau + pYES-DEST52-BMEI1305 | pGAL1-omp2b-V5-His6                           | Sc-Ura          | This study            |
| GL0003     | BF264-15 Dau + pYES-DEST52-BMEI0660 | pGAL1-fecE-V5-His6                           | Sc-Ura          | This study            |
| GL0004     | QX95001 + pYES-DEST52-BMEI1305   | pGAL10-bax + pGAL1-omp2b-V5-His6               | Sc-Leu-Ura      | This study            |
| GL0005     | QX95001 + pYES-DEST52-BMEI0660   | pGAL10-bax + pGAL1-fecE-V5-His6                | Sc-Leu-Ura      | This study            |
| GL0007     | QX95001 + pYES-DEST52-BMEI1306   | pGAL10-bax + pGAL1-omp2b-V5-His6               | Sc-Leu-Ura      | This study            |
| GL0008     | QX95001 + pYES-DEST52-BMEI1249   | pGAL10-bax + pGAL1-omp25-V5-His6               | Sc-Leu-Ura      | This study            |
| GL0010     | BF264-15 Dau + pYES-DEST52-t-omp2b | pGAL1-t-omp2b-V5-His6                      | Sc-Ura          | This study            |
| GL0011     | QX95001 + pYES-DEST52-t-omp2b    | pGAL10-bax + pGAL1-t-omp2b-V5-His6             | Sc-Leu-Ura      | This study            |
| GL0012     | BF264-15 Dau + pYES-DEST52-short-omp2b | pGAL1-short-omp2b-V5-His6              | Sc-Ura          | This study            |
| GL0013     | QX95001 + pYES-DEST52-short-omp2b | pGAL10-bax + pGAL1-short-omp2b-V5-His6        | Sc-Leu-Ura      | This study            |
| GL0014     | BF264-15 Dau + pYES-DEST52-BMEI1306 | pGAL1-omp2b-V5-His6                       | Sc-Ura          | This study            |
| GL0015     | BF264-15 Dau + pYES-DEST52-BMEI1249 | pGAL1-omp25-V5-His6                     | Sc-Ura          | This study            |
| GL0016     | QX95001 + pYES-DEST52          | pGAL10-bax + empty destination vector        | Sc-Leu-Ura      | This study            |
| GL0017     | QX95001 + pYES-DEST52-long-omp2a | pGAL10-bax + pGAL1-long-omp2a-V5-His6        | Sc-Leu-Ura      | This study            |
| GL0018     | BF264-15 Dau + pYES-DEST52-long-omp2a | pGAL1-long-omp2a-V5-His6                | Sc-Ura          | This study            |

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116 clones tested, only two yeast strains, expressing \textit{bax} and either \textit{B. melitensis} ORF BMEI1305 or BMEI0660, reproducibly grew as well as the control GL0001 strain (Figure 1A). These ORFs encode Omp2b and FecE proteins, respectively. FecE is predicted to be a cytoplasmic ATP-binding protein associated with other components of an iron (III) ABC transporter. Omp2b is described as a porin [25]. The production of both Omp2b and FecE in yeast (GL0002 and GL0003 strains, respectively) was checked by Western blot analysis (Figure 1B). The growth phenotype conferred by Omp2b or FecE is not linked to an absence or decrease of Bax production as Bax was detected in yeast expressing \textit{bax} and \textit{omp2b} or \textit{fecE} (GL0004 and GL0005). Thus, our screen identified two \textit{B. melitensis} proteins, Omp2b and FecE, of which the expression in yeast prevents cell death induced by the mammalian pro-apoptotic protein Bax. FecE mechanism of action regarding the inhibition of Bax-induced yeast lethality remains to be investigated but may be related to its ATPase activity, considering the various implications of ATPases in intracellular signaling pathways. However we cannot exclude that FecE effect in yeast is independent of this predicted function. Nevertheless, the ability of FecE to act as an anti-apoptotic effector should be tested in the future in other models than the yeast-based system used here as a screening tool.

Because bacterial porins were already reported to be involved in apoptosis modulation [26,27,28], this study focuses on the second candidate, Omp2b. Outer membrane proteins (OMP) harbor a N-terminal signal peptide (SP) for the recognition and cleavage of the unfolded proteins by the Sec system before their secretion in the periplasm and the folding and insertion of the mature proteins into the bacterial outer membrane [29]. In the \textit{B. melitensis} ORFeome, the deduced sequence from \textit{omp2b} ORF contains a long predicted SP (PSP). A truncated form of Omp2b lacking the entire PSP (t-Omp2b) only poorly inhibits Bax effects in yeast (GL0011) when compared with negative and positive control strains GL0007 and GL0004 (Figure 2A and 2B). This lower protection against Bax lethal effects is not related to a toxicity induced by t-Omp2b since a strain lacking \textit{bax} and producing t-Omp2b (GL0010) grows similarly as the GL0004 strain that produces Omp2b (data not shown). In addition, a variant of Omp2b harboring a N-terminally shortened SP (short-Omp2b) completely looses the anti-Bax effect (GL0013), indicating that the production of Omp2b with its complete PSP is required for a strong Bax inhibition in yeast (Figure 2A and 2B). Interestingly, Omp2b and t-Omp2b are detected on Western blot at a similar apparent molecular weight, which is lower than the one observed for short-Omp2b (Figure 2C), suggesting that Omp2b PSP is cleaved in yeast, unlike the shortened PSP of the short-Omp2b form. Notably, t-Omp2b was reproducibly detected in a much lesser quantity in Western blot when compared with Omp2b (Figure 2C), which could account for its weaker “anti-Bax” effect. This might suggest that the PSP, and/or the PSP cleavage event, are required for Omp2b production or stability leading to protein levels that are sufficient to remarkably inhibit Bax lethal effects. Altogether, our results support the requirement of PSP cleavage for Omp2b anti-Bax effect in yeast. The processing of Omp2b PSP in yeast is not surprising considering...
previous studies showing the functionality of bacterial SP in eukaryotic cells [30,31,32]. We hypothesize that the processed mature form of Omp2b could adopt a fold allowing its particular subcellular localization or interaction with physical partners, which might lead to the inhibition of Bax-induced effects. This is supported by the Western blot detection of a high molecular weight form of Omp2b in a GL0002 strain protein extract, in semi-denaturing conditions, at a size expected for the trimeric assembly of the porin (data not shown).

Only two ORFs encode porin homologs (Omp2a and Omp2b) in the B. melitensis genome. Omp2b is encoded on the opposite strand and next to BME11306 ORF encoding Omp2a. Omp2b shares 83% identity with Omp2a. To assess the specificity of Omp2b in the inhibition of Bax-induced cell death in yeast, we tested the effect of Omp2a, but also of Omp25, a major B. melitensis outer membrane proteins (OMP), on the growth of yeast expressing bax (GL0007 and GL0008, respectively). None of them (3 independent clones tested by strain) was able to rescue yeast from Bax-induced cell death (Figure 3). The production of those proteins in GL0007 and GL0008 has been verified by Western blot (Figure S1 of Supporting information), suggesting that their inability to rescue yeast from Bax-induced cell death is not due to the absence of the OMPs. Their expression had no effect on the growth of the wild type yeast strain BF264-15 Dau (GL0014 and GL0015, data not shown), arguing against their hypothetical toxicity for this strain. Thus, the “anti-Bax” effect of Omp2b is specific to this OMP, since it is not even observed for Omp2a although both porins share high sequence similarity. Omp2a predicted signal peptide is identical to the shortened PSP of short-Omp2b. To determine if the functional difference between Omp2a and Omp2b can be explained by the difference in signal peptides, we constructed a version of Omp2a, in which the predicted signal peptide sequence has been replaced by the full Omp2b PSP (long-Omp2a). When expressed in yeast (GL0017), long-Omp2a was not able to prevent Bax-induced cell death, suggesting that although Omp2b PSP is required for Omp2b effect in yeast, other features in the porin are necessary and not shared by Omp2a (Figure S2 of Supporting information). B. melitensis Omp2a was shown to form a pore that is more efficient in sugar diffusion than Omp2b [25]. Whether the pore features are
necessary for the function leading to the rescuing effect is presently unknown. Characteristic porin structures are composed of beta-barrels, with short periplasmic turns and external loops of variable length [33]. Interestingly, the external L3 and L5 loops are enriched in major structural differences between Omp2a and Omp2b [25], as between PorB porins from Neisseria meningitidis and gonorrheae, which have been characterized as showing opposite effects regarding host apoptosis [26,27,28].

Among other hypotheses on Omp2b mechanism of action in yeast, it would be tempting to speculate that the porin, once processed, might associate with mitochondria in yeast, given (i) the involvement of this organelle in Bax-induced lethality, (ii) the mitochondrial localization of neisserial PorB in infected cells [26,27,34], (iii) that ectopically produced bacterial β-barrels lacking their SP have been shown to locate to yeast mitochondria, by an import pathway shared with mitochondrial beta-barrels, involving import receptors and the TOB/SAM complex [35]. Alternatively, it is conceivable that Omp2b, in the cytosol, might prevent Bax mitochondrial targeting, either indirectly or by interacting with Bax directly. A pull-down experiment to test if Bax and Omp2b physically interact in yeast could not be performed since Omp2b is found in the insoluble fraction of lyzed yeast cells, which is not surprising considering its predicted tridimensional structure exposing hydrophobic side chains [25]. Nevertheless, it is completely conceivable that Omp2b prevents yeast cell death indirectly, that is without a physical interaction with Bax, taking into account the pleiotropic effects of Bax that have been proposed to be involved in yeast cell death [11].

This study is based on a screening method involving a functional assay in a "synthetic" system. Although several proteins previously identified by this strategy have been demonstrated to be involved in cell death inhibition in superior eukaryotic systems [14,15,16,17,18], Omp2b and FecE should be characterized further in a model that is more relevant to the natural infection conditions, such as transfection of constructs carrying these coding sequences in mammalian cells. Determining the role of Omp2b during infection should also rely on a reverse genetics approach aiming at the comparison of wild type and omp2b deletion strains of B. melitensis in their ability to inhibit apoptosis of infected cells. However, this was recluded by the essentiality of omp2b gene in B. melitensis (data not shown). The observation that Omp2a production in yeast does not induce a rescuing effect like Omp2b suggests that these differences between Omp2a and Omp2b might be related not only to porin functions but also to differences in apoptosis modulation.

The possible effect of Omp2b inside infected cells might be dependent on Omp2b delivery by outer membrane vesicles (OMV) that have been shown to harbor Omp2b [36] and serve as a secretion mechanism for effectors of other bacteria [37], or by the release of outer membrane fragments. It was indeed reported that Brucella LPS may travel to the host cell surface, supporting trafficking involving outer membrane components in infected cells [38,39].

To the best of our knowledge, this study illustrates the first application of a screening strategy for the identification of Bax inhibitors in yeast to a bacterial genome. The proteins identified are anti-apoptotic effector candidates, of which the possible modulator effect on apoptosis can be characterized in the future in various mammalian cellular models of apoptosis.

Materials and Methods

Features and references for the yeast and bacterial strains used in this study are available in Table 1. Primers sequences are available in Table S1 of Supporting information section. The construction and screening of the ‘‘bax+ORFeome’’ yeast library is described in details in the Supporting information section.

Plasmids

pYES-DEST52-BMEI1305 (omp2b), pYES-DEST52-BMEI0660 (fecE), pYES-DEST52-BMEI1306 (omp2a), pYES-DEST52-BMEI1249 (omp2b) and pYES-DEST52-bclXL were obtained after LR recombination reaction (Gateway, Invitrogen) between pYES-DEST52 destination vector (Invitrogen 12286-019) and each corresponding pDONR201-ORF from the B. melitensis 16M ORFeome [23] or pDONR201-bclXL. pDONR201-bclXL was obtained after a BP recombination reaction (Gateway, Invitrogen) between pDONR201 and an amplification product of human bclXL DNA lacking the stop codon (from pcDNA3myc-bclXL, kindly provided by T. Arnould, University of Namur, Belgium) flanked by attB1 and attB2 recombination sites (primers attB1-bclXL and attB2-bclXL). t-omp2b and short-omp2b were amplified from pYES-DEST52-omp2b with t-omp2b-attB1 and t-omp2b-attB2 primers, and from pYES-DEST52-omp2a with FWDshortomp2b and t-omp2b-attB2 primers, respectively. Each amplification product was cloned in pDONR201 by BP reaction. Resulting entry vectors were LR recombined with pYES-DEST52. Construction of pYES-DEST52-long-omp2a was based on a 2-steps PCR strategy. Fragment A was amplified from pYES-DEST52-omp2b with primers long-omp2a_1 and long-omp2a_2. Fragment B was amplified from pYES-DEST52-omp2a with primers long-omp2a_3 and long-omp2a_4. long-omp2a was amplified by overlap extension PCR with fragment A and B as matrixes, and with primers long-omp2a_1 and

Figure 3. The Bax-suppressor effect of Omp2b is not common to B. melitensis outer membrane proteins. Drops of serial dilutions of saturated cultures, on media allowing repression or induction of bax and ORF expression, for GL0008 (omp25) and GL0007 (omp2a). GL0001 (bclXL) and GL0004 (omp2b) strains are positive controls for growth. Dilution factors are indicated. Pictures were taken after 4 days of growth at 30°C. doi:10.1371/journal.pone.0013274.g003
Omp2a and Omp25 coding sequences are fused with a His6 tag in except for Bax detection.

Total protein extracts from *S. cerevisiae*

Extraction protocol was modified from [40] with 4% SDS in the SDS sample buffer.

**Antibodies used in Western blots**

Bax was detected using HRP-coupled anti-Bax antibody (Santa Cruz sc-493 HRP), diluted 1:100 in PBS-1% non-fat milk. Phosphoglycerate kinase was detected using mouse monoclonal anti-PGK antibody (Invitrogen Cat. No. A-6457), diluted 1:10,000 in PBS-1% non-fat milk. Hiss-tagged fusions were detected using anti-6-His mouse monoclonal antibody (Covance Cat. No. MMS-156R), diluted 1:1,000 in PBS-1% non-fat milk. Omp2b and variants described in this study were detected using monoclonal A6304D11G01 mouse antibody [25] as primary antibody. Tubulin was detected using an anti-mouse monoclonal anti-alpha tubulin antibody (Sigma-Aldrich Cat. No. T5168) diluted 1:50,000 in PBS-1% non-fat milk. Anti-mouse IgG, HRP-linked antibody (Amersham Cat. No. NA931) was used at 1:5000 diluted in PBS-1% non-fat milk. Omp2a was detected using monoclonal anti-6-His mouse monoclonal antibody (Covance Cat. No. MMS-156R), diluted 1:1,000 in PBS-1% non-fat milk. Omp2b and variants described in this study were detected using monoclonal A6304D11G01 mouse antibody [25] as primary antibody. Tubulin was detected using an anti-mouse monoclonal anti-alpha tubulin antibody (Sigma-Aldrich Cat. No. T5168) diluted 1:50,000 in PBS-1% non-fat milk. Anti-mouse IgG, HRP-linked antibody (Amersham Cat. No. NA931) was used at 1:5000 diluted in PBS-1% non-fat milk. Omp2a was always used as secondary antibody except for Bax detection.

**Supporting Information**

**Figure S1** Western blot showing that Omp2a or Omp25 is produced in BF264-15Dau yeast clones transformed with pYep51-bax and pYES-DEST52-omp2a (GL0007) or pYES-DEST52-omp25 (GL0008). Three clones were tested for each strain. The detection was made with a monoclonal anti-His, since Omp2a and Omp25 coding sequences are fused with a His tag in the pYES-DEST52 plasmids. The expected sizes of Omp2a and Omp25 fused to His6 are 43.9 and 27.6 kDa, respectively. Bax was detected with an anti-Bax antibody. The expected size of Bax is 21.2 kDa.

**References**