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A bacterial two-hybrid genome fragment library for deciphering regulatory networks of the opportunistic pathogen *Pseudomonas aeruginosa*

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Bacterial gene regulation is controlled by complex regulatory cascades which integrate input environmental signals and adapt specific and adequate output cellular responses. These complex networks are far from being elucidated, in particular in *Pseudomonas aeruginosa*. In the present study, we developed bacterial two-hybrid genome fragment libraries of the *P. aeruginosa* PAO1 strain to identify potential partners involved in the HptB/HsbR/HsbA pathway. This powerful tool, validated by the interaction previously described between HsbR and HsbA proteins, allowed us to demonstrate that the HsbR response regulator dimerizes through its PP2C/ATPase C-terminal effector domain, an observation further confirmed by pull-down experiments. This will also allow us to identify further new partners in this cascade.

INTRODUCTION

Bacteria have evolved complex regulatory networks to specifically lock or switch on and modulate the expression of a series of genes, thus leading to a highly specific and adequate cellular response to particular environmental signals. These regulatory cascades integrate environmental signals through appropriate sensing systems, forming complex networks that include two-component systems (TCSs), transcriptional regulators, quorum sensing and post-transcriptional regulatory mechanisms (Bordi & de Bentzmann, 2011). Among them, the TCSs, which represent a predominant signalling mechanism in bacteria, monitoring external and internal stimuli (e.g. nutrients, ions, temperature, redox states, etc.), translate these signals into adaptive responses. The TCS GacS/GacA in *Pseudomonas aeruginosa* is a good example of how this human pathogen regulates the expression of quorum sensing and of several virulence factors (Burrowes *et al.*, 2006; Heurlier *et al.*, 2004; Kay *et al.*, 2006; Pessi *et al.*, 2001; Rahme *et al.*, 2000; Reimmann *et al.*, 1997) through the expression of two small RNAs (sRNAs), RsmY and RsmZ (Brencic *et al.*, 2009). This TCS pathway is itself modulated by two accessory hybrid histidine kinases, RetS (Goodman *et al.*, 2004) and LadS (Ventre *et al.*, 2006), and probably by a plethora of mostly unknown protein factors. The complexity of this regulatory pathway has recently increased through the characterization of a new player, the HptB signalling pathway (Bordi *et al.*, 2010). Thus, RetS and HptB cross-connect at the level of the GacS/GacA pathway to control sRNAs RsmY and RsmZ and RsmY production alone, respectively (Bordi *et al.*, 2010), and therefore are not cognate partners in this cascade. Additionally, the HptB protein has been demonstrated to interact with the *PA3346* gene product (Bordi *et al.*, 2010; Hsu *et al.*, 2008). This gene encodes a response regulator (RR) composed of an N-terminal phosphoryl-receiver domain (D), and a C-terminal output domain (PP2C/ATPase) containing a phosphatase 2C (PP2C) subdomain (Delumeau *et al.*, 2004) and an additional possible ATPase subdomain. This RR has been renamed HsbR for HptB-dependent secretion and biofilm regulator. Using the bacterial two-hybrid technique, we previously demonstrated that HptB interaction occurs with the HsbR D domain of the RR, but not with its PP2C/ATPase domain. Furthermore, we showed that the PP2C/ATPase domain, but not the D domain of HsbR, interacts with the *PA3347* gene product. The putative PA3347 product has similarities to a putative anti anti-σ factor (Bordi *et al.*, 2010), and has been renamed HsbA for HptB-dependent secretion and biofilm anti anti-σ factor. Interestingly, the *hptB*, *PA3346* and *PA3347* genes form a polycistronic operon (Hsu *et al.*, 2008). As the partners of this cascade, in particular those downstream of the HptB/HsbR/HsbA node directly controlling *rsmY* expression, are all far from being identified, with candidates probably dispersed throughout the genome, we designed a pan-genomic strategy using the bacterial two-hybrid technique.

The yeast two-hybrid method was initially developed by Fields & Song (1989). The bacterial two-hybrid variant was further developed to characterize interactions between two targeted proteins (Karimova *et al.*, 1998). It has been extended to screen for previously unknown partners

Abbreviations: Ap, ampicillin; Km, kanamycin; RR, response regulator; sRNA, small RNA.

involved in antibiotic resistance (Domain *et al.*, 2007) and bacterial viability (Handford *et al.*, 2009) in *Escherichia coli*, in spore wall synthesis in *Streptomyces coelicolor* (Kleinschnitz *et al.*, 2011) and in virulence in *Mycobacterium tuberculosis* (Klepp *et al.*, 2009) by high-throughput testing of interactions between a bait protein of interest and pan-genomic preys. Additionally, a modified version using Zif and RNAPω domains permitted the development of a two-hybrid library system based on Gateway for *Vibrio cholerae* and *Francisella tularensis* (Karna *et al.*, 2010). A yeast two-hybrid genome fragment library of the PAO1 strain has been very recently developed and screened with phage early infecting proteins as baits to identify interacting proteins of *P. aeruginosa* (Roucourt *et al.*, 2009). The yeast two-hybrid system is based on the reconstruction of the Gal4p transcription factor, which once reconstituted, binds DNA and activates expression of the reporter gene. This reconstruction needs to occur in the vicinity of the transcriptional machinery, and therefore implicates the entry of candidate partners into the nucleus, which is a limitation of the technique. A second limitation of the yeast two-hybrid system is represented by the absolute and precise requirement for spatial positioning of the two Gal4p domains for correct DNA binding and for reporter gene induction, therefore leading to a size limitation of the bait and the prey for the interaction tests. Thus, the bacterial two-hybrid method is suitable to bypass these limitations and presents several specific advantages. Based on the reconstitution of the catalytic domain (formed of T18 and T25 subdomains) of the CyaA adenylate cyclase of *Bordetella pertussis* (Karimova *et al.*, 1998), it is easier to manipulate (the doubling time being shorter). Moreover, reconstitution of a CyaA-dependent activity leads to synthesis of cAMP, a molecule which can be spatially uncoupled from transcriptional events. Additionally, the bacterial two-hybrid method is able to detect protein interactions that occur either in the cytosol or in the inner membrane. From the species point of view, bacterial protein–bacterial protein interactions would probably be detected with higher efficacy in bacteria (even if the two-hybrid screen is performed in a heterologous host such as *E. coli*), in particular due to the possible cytotoxic nature of the produced proteins.

Therefore, in the present study, we developed bacterial two-hybrid genome fragment libraries of the *P. aeruginosa* PAO1 strain, which confirmed the interaction between the C-terminal effector domain (PP2C/ATPase domain) of the RR HsbR and the HsbA putative anti anti-σ factor. We also extended the characterization of the RR HsbR by showing that this regulator dimerizes not only through its N-terminal domain (Bordi *et al.*, 2010) but also through its C-terminal effector domain, a dimerization mechanism which was confirmed using pull-down experiments.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in the study are described in Table 1. Bacteria were grown aerobically in Luria–Bertani (LB) broth or LB agar at 37 or 30 °C. To visualize bacterial two-hybrid interactions on solid medium, MacConkey indicator medium (Difco) supplemented with maltose (final concentration 1 %), IPTG (100 μM), ampicillin (Ap; 100 μg ml^{-1}) and kanamycin (Km; 50 μg ml^{-1}), herein designated MacConkey medium, was used. When needed, the liquid cultures were supplemented with IPTG at a final concentration of 100 or 500 μM. Ap (100 μg ml^{-1}), tetracycline (Tc; 15 μg ml^{-1}) and Km (50 μg ml^{-1}) were added to maintain the plasmids.

Two-hybrid plasmid construction. DNA fragments encoding protein domains of interest were cloned at the 5′ end of genes encoding the two fragments T25 and T18 of *B. pertusssis* Cya adenylate cyclase carried on the pKT25 and pUT18C vectors, respectively. pUT18C-HsbRPP2C/ATPase, pKT25-HsbA and pUT18C-HsbRD were constructed from PAO1 DNA with oligonucleotides used previously (Bordi *et al.*, 2010). The DNA regions encoding the receiver domain (D) or the PP2C/ATPase domain of HsbR were amplified by PCR using oligonucleotides listed in Table 2, digested with *Xba*I and *Kpn*I enzymes and cloned into the pKT25 vector, yielding, respectively, pKT25-HsbRD and pKT25-HsbRPP2C/ATPase. The absence of mutation was checked by sequencing. An adenylate cyclase-deficient *E. coli* strain, BTH101, was used to screen for positive interactions. BTH101 competent cells were transformed simultaneously with pKT25 and pUT18 derivatives, and co-transformants were selected on agar plates supplemented with Ap (100 μg ml^{-1}) and Km (50 μg ml^{-1}). Single colonies were spotted on MacConkey medium. Positive interactions were identified as red colonies after a 24–48 h period of incubation at 30 °C.

Generation of *P. aeruginosa* two-hybrid genome fragment libraries. The genomic DNA of *P. aeruginosa* strain PAO1 was prepared using PureLink Genomic DNA kit (Invitrogen) and partially digested by *Sau*3A. The randomly digested DNA was separated on a 0.8 % agarose gel, and fragments ranging in size from 150 to 2000 bp were gel-purified using a Qiagen gel extraction kit (Qiagen).

Four independent two-hybrid libraries were separately constructed. The first two libraries were generated using the pUT18 and the pUT18C vectors (Karimova *et al.*, 1998). The other two libraries used pUT18-derivative vectors in which the polylinker site was shifted to +1 and +2 nt, respectively (Handford *et al.*, 2009). The vectors pUT18C, pUT18, pUT18+1 and pUT18+2 were digested by *Bam*HI (New England Biolabs) for 2.5 h at 37 °C and dephosphorylated with Shrimp Alkaline Phosphatase (New England Biolabs) for 1 h at 37 °C. Several independent pools of DNA fragments were ligated overnight at 16 °C into the different pUT18 linearized vectors using T4 ligase (Roche). The resulting ligation mixture was transformed into *E. coli* DH5α competent cells, and plated on LB-Ap plates. The resulting transformants were collected by scraping the plates with a glass spreader, and pooled before plasmid isolation (PureYield Plasmid Midiprep System, Promega). The quantity of each final plasmid library was evaluated under UV illumination.

The mean size insert being 769 bp, one fold coverage was therefore estimated to require 48 877 clones. Therefore, the estimation of genome coverage for each library and of total libraries constructed was calculated and is presented in Table 3. Further, the probability of having a particular fragment cloned in the library was calculated using the following formula:

$$p = 1 - (1 - \frac{i}{G})^N$$

Where i is the mean insert size, G the genome size and N the number of clones obtained in the library (Clarke & Carbon, 1976). Further calculation of the probability of having such a fragment inserted in the right orientation and in-frame with the *cyaA* T18 fragment was performed by multiplying G by 6.

Table 1. Strains and plasmids used in this study

Strain, plasmid or library	Genotype or description	Reference or source
***E. coli* strains**		
BTH101	F$^-$ *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*	Karimova *et al.* (2001)
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* (ϕ17;80*lacZ* ΔM15)	Laboratory collection
TG1	*supE* Δ(*lac-proAB*) *thi hsdR*Δ*5* (F′ *traD36 rpoA*$^+$ *B*$^+$ *lacI*^{q} *Z*ΔM15)	Laboratory collection
Plasmids		
pCR2.1	ColE1, f1 ori, Ap^R, Km^R	Invitrogen
pUT18	Vector encoding T18 fragment of *B. pertussis cyaA*; Amp^R	Karimova *et al.* (2001, 1998)
pUT18 + 1	Modified version of pUT18 with reading frame shifted to + 1	Handford *et al.* (2009)
pUT18 + 2	Modified version of pUT18 with reading frame shifted to + 2	Handford *et al.* (2009)
pKT25	Vector encoding T25 fragment of *B. pertussis cyaA*; Km^R	Karimova *et al.* (2001)
pUT18C	Modified version of pUT18 with the polylinker located on the C-terminal end of T18	Karimova *et al.* (2001)
pBBRMCS3	Broad-host-range vector, IncP; *lacZ*α Tc^R	Laboratory collection
pKT25-HsbA	pKT25 plasmid containing *cyaAT25–hsbA* fusion	This study
pUT18C-HsbA	pUT18C plasmid containing *cyaAT18–hsbA* fusion	This study
pUT18C-HsbRPP2C/ATPase	pUT18C plasmid containing *cyaAT18–hsbRPP2C/ATPase* domain fusion	This study
pKT25-HsbRPP2C/ATPase	pKT25 plasmid containing *cyaAT25–hsbRPP2C/ATPase* domain fusion	This study
pUT18C-HsbRD	pUT18C plasmid containing *cyaAT18–hsbRD* receiver domain fusion	This study
pUT18C-HsbRlib	pUT18C plasmid isolated from library screen and containing a truncated *hsbR* fused to T18	This study
pCR2.1*StrephsbRlib*	*hsbRlib* DNA fragment N-fused to Strep tag cloned in pCR2.1	This study
pBBR*FLAGhsbRlib*	*hsbRlib* DNA fragment N-fused to FLAG tag cloned at *Xba*I/*Sac*I sites in pBBRMCS3	This study
PAO1 libraries		
pUT18CPAO1Lib	pUT18C containing PAO1 genomic library as a C-terminal T18 fusion	This study
pUT18PAO1Lib	pUT18 containing PAO1 genomic library as an N-terminal T18 fusion	This study
pUT18 + 1PAO1Lib	pUT18 + 1 containing PAO1 genomic library as an N-terminal T18 fusion	This study
pUT18 + 2PAO1Lib	pUT18 + 2 containing PAO1 genomic library as an N-terminal T18 fusion	This study

High-throughput two-hybrid assays. Libraries were tested independently against each pKT25 bait (pUT18CPAO1Lib for *hsbA* and pUT18CPAO1Lib, pUT18PAO1Lib, pUT18 + 1PAO1Lib and pUT18 + 2PAO1Lib for *hsbR*). Basically, 25–50 ng of each pUT18-derivative library was transformed into 100 µl of electrocompetent BTH101 cells carrying the pKT25 bait vector and plated on MacConkey medium for 48–96 h at 30 °C. Red colonies were picked up and restreaked on MacConkey plates. The colonies remaining red after replating were recultivated in liquid medium, and plasmids were isolated using a QIAprep Spin Miniprep kit (Qiagen) and further analysed. The

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)
Double hybrid construction	
hsbA	GCTCTAGAGATGGCCATCACTGCGCTGCCGGGGTACCTCAGCTGATCTTGAACAACTGC
hsbRPP2C/ATPase	GCTCTAGACAACGTGCGCTACCTGCAATCGCCGAGCTCCGACGAAAGCGCGATTATGCCTGAGG
hsbRD	GCTCTAGATGAGATGAATCCCCCGGCGGAACGGAATTCTCACGGCGAGAAATACTGGTTTCG
Sequencing of prey	
pUT18CUp	CGGATGTACTGGAAACGGTG
pUT18NDn	TGCGGAACGGGCGCCGGCGCGAGCG
Pull-down experiments	
*FLAG-hsbRlib*Up	GCTCTAGAGAGCGCAAGAACTGATGGACTATAAAGACGACGACGACAAAAGCAACGTGCGCTACCTGCAATCG
*Strep-hsbRlib*Up	GCTCTAGAGAGCGCAAGAACTGATGTGGAGCCACCCGCAGTTCGAAAAAAGCAACGTGCGCTACCTGCAATCG
*hsbRlib*Dn	CCGAGCTCAAGCGCGATTATGCCTGAGG

Table 3. Characteristics of PAO1 genome fragment libraries constructed in this study

The calculation of genome coverage was done as follows. The average size of the insert was estimated at 769 bp. Considering that the genome size of strain PAO1 is 6 264 404 bp and that the probability of obtaining an insert cloned in-phase is 1/6, the number of clones required to cover the genome would be 48 877 clones. Considering that we obtained 140 000 clones for the pUT18CPAO1Lib library and that 68.2 % of the clones have an insert (150–2000 bp), we estimated that 95 486 clones were positive and that the coverage of the pUT18CPAO1Lib library was 95 480/48 877, i.e. about 2. P_1, Probability of having a particular fragment cloned in the library calculated as described in Clarke & Carbon (1976); P_2, probability of having a particular fragment cloned in-phase with the *cyaA* T18 fragment in the library.

Library	Approximate total no. of clones	Percentage of clones with an insert (150–2000 bp)	No. of clones with insert	PAO1 genome coverage	P_1	P_2
pUT18CPAO1Lib	140 000	68.2	95 486	2.0 ×	0.99	0.86
pUT18PAO1Lib	24 900	71.4	17 778	0.4 ×	0.89	0.31
pUT18 + 1PAO1Lib	23 000	73.9	16 997	0.3 ×	0.87	0.29
pUT18 + 2PAO1Lib	23 100	71.4	16 493	0.3 ×	0.87	0.29
Final library	211 000	71.2	146 748	3.0 ×	0.99	0.95

corresponding isolated plasmids (pKT25 bait and pUT18 derivative prey plasmids) were transformed again in DH5α cells, and co-transformants were plated on Ap plates to cure the pKT25 plasmid. Transformants that were Ap^R Km^S were selected in order to isolate only the pUT18 prey plasmids. The candidate preys were retested individually for interaction with the bait by retransforming pUT18 derivative prey and pKT25 bait plasmids into BTH101 cells but also in parallel to pUT18 derivative prey and the pKT25 empty vector. The interaction was evaluated by the colour of spotted co-transformants on MacConkey plates and by the measurement of their resulting β-galactosidase activities.

β-Galactosidase assay. The co-transformants of interest were grown in liquid LB medium at 37 °C at 250 r.p.m. for 16 h. A 500 µl volume of each culture was pelleted and mixed with 900 µl Z buffer (per litre: 10.7 g $Na_2HPO_4.2H_2O$, 5.5 g NaH_2PO_4, 0.75 g KCl, 0.246 g $MgSO_4.7H_2O$, 2.7 ml β-mercaptoethanol, pH 7) before addition of 20 µl of 0.1 % (w/v) SDS and 100 µl $CHCl_3$ for permeabilization. A 40 µl volume of ONPG solution (4 mg ml^{-1} in Z buffer without β-mercaptoethanol) was added to 20 µl of permeabilized cells diluted in 180 µl Z buffer, and β-galactosidase activity was then calculated and expressed in Miller units (Thibodeau *et al.*, 2004). All assays were performed in triplicate and results are expressed as means ± SDs.

Pull-down experiments. A DNA fragment corresponding to HsbRlib was amplified with an N-terminal FLAG or Strep tag, and cloned into vector pCR2.1. The sequence was checked and the *FLAG-hsbRlib* DNA fragment was further digested with *Xba*I/*Sac*I for subcloning in vector pBBRMCS3. Production of FLAG-HsbRlib and Strep-HsbRlib versions was checked in *E. coli* TG1 cells. Both vectors were introduced into *E. coli* in parallel to transformation with the corresponding empty vector, so that the following combinations were further examined: pCR2.1/pBBR*FLAGhsbRlib*, pCR2.1/pBBRMCS3, pCR2.1*StrephsbRlib*/pBBR*FLAGhsbRlib*, pCR2.1*StrephsbRlib*/pBBRMCS3. Fifty millilitres of cells were cultured with 500 µM IPTG, stopped in the exponential growth phase (corresponding to OD_{600} 1) and resuspended in 10 mM Tris buffer supplemented with cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche) and 50 mM NaCl. One hundred micrograms of DNase and RNase were added and cells were lysed by French press. Total lysates were mixed with 50 µl agarose beads coupled with antibody against FLAG peptide (Sigma) and incubated on a rotating wheel for 1 h at room temperature. The unbound fraction was collected by centrifugation for 2 min at 2000 r.p.m. Beads were washed twice with 1 ml of 10 mM Tris buffer supplemented with cOmplete, EDTA-free Protease Inhibitor Cocktail and 50 mM NaCl, and were collected by centrifugation, resuspended in loading buffer and heated for 10 min at 95 °C before analysis by SDS-PAGE and immunoblotting.

SDS-PAGE and Western blotting. Beads corresponding to 10 OD_{600} equivalent units for each condition were loaded in parallel to 0.3 OD_{600} equivalent unit for total cells producing only one tagged HsbRlib version. Proteins were separated by electrophoresis in a 12 % polyacrylamide gel. FLAG-HsbRlib and Strep-HsbRlib proteins were immunodetected with the monoclonal antibody against the FLAG epitope (1 : 2000, Sigma) and with StrepTactin and Alkaline Phosphatase (AP) conjugate (1 : 4000, IBA), respectively. A secondary horseradish peroxidase-coupled anti-mouse antibody (1 : 5000, Sigma) was further used to reveal the FLAG signal.

RESULTS AND DISCUSSION

Four different *P. aeruginosa* two-hybrid genome fragment libraries were constructed in the present study (Table 3). The first one, designated pUT18CPAO1Lib, consisted of approximately 140 000 clones, which represents twofold coverage of the PAO1 genome. In order to get a large coverage of the PAO1 genome, we engineered three additional libraries (pUT18PAO1Lib, pUT18 + 1PAO1Lib and pUT18 + 2PAO1Lib) in which genome fragment libraries were cloned at the N-terminal part of the T18 domain in the pUT18 vector or shifted to + 1 and + 2 nt in the pUT18 + 1 and pUT18 + 2 vectors, respectively. Each of these additional libraries contained 23 000 to 25 000 clones and represents onefold coverage in total of the genome. Thus, the final PAO1 prey libraries consisted of 211 000 clones, and were estimated to represent threefold coverage of the PAO1 genome. The number of clones is comparable with those obtained for most two-hybrid libraries constructed for other bacteria (Domain *et al.*, 2007; Handford *et al.*, 2009; Klepp *et al.*, 2009) but is smaller than that obtained for *S. coelicolor* (Kleinschnitz *et al.*, 2011). To characterize the diversity of the library inserts, a total of 101 transformants were arbitrarily chosen from the four different libraries. PCR and/or restriction analysis showed that more than 70 % of

these pUT18-derivative plasmids had a PAO1 genome fragment inserted, a percentage similar to those obtained in the *P. aeruginosa* yeast library (Roucourt *et al.*, 2009) but smaller than the percentage obtained in *S. coelicolor* (Kleinschnitz *et al.*, 2011). As expected, the insert length varied between 150 and 2000 bp, confirming that our PAO1 fragment generation protocol was adequate. Among the inserts, 74 % showed a length between 150 and 1000 bp and 26 % a length between 1000 and 2000 bp. The average fragment size was found to be 769 bp.

We previously showed that in *P. aeruginosa*, the non-coding sRNA RsmY is controlled by the HtpB/HsbR/HsbA regulatory pathway (Bordi *et al.*, 2010). Using a targeted bacterial two-hybrid approach, we previously showed that the histidine phosphotranfer B protein (HptB) interacts with the D receiver domain of the RR HsbR. The effector domain of HsbR, composed of a PP2C module and a possible ATPase subdomain, has been described as interacting specifically with the putative anti anti-σ factor HsbA (Bordi *et al.*, 2010). We therefore used the HsbA and HsbR proteins of this regulatory pathway to test our high-throughput *P. aeruginosa* two-hybrid bacterial library with the aim of identifying unknown partners. The pKT25-HsbA bait construct was combined with the *P. aeruginosa* PAO1 fragment prey library constructed in the pUT18C vector, and the number of co-transformants obtained on MacConkey medium reached 349 000 clones. Among the co-transformants, 68 red clones were identified, including 35 strongly red clones. These 35 strongly red clones, which probably contained DNA encoding putative prey for HsbA, were retested for interaction on MacConkey medium by individually retransforming the different pUT18 derivative preys and the pKT25-HsbA or pKT25 empty vector plasmids into BTH101 cells. Among co-transformants, 30 were still red on MacConkey medium, and the DNA sequence inserts contained in the pUT18 derivative vectors were further identified by sequencing. In eight clones out of 30 (~27 %), the prey fragment corresponded to an extended peptide sequence, further designated HsbRlib (peptide fragment from amino acid 175 to amino acid 571), of the predicted effector domain (amino acid 186 to amino acid 571) of the RR HsbR (Fig. 1).

The pKT25-HsbRPP2C/ATPase bait construct was also combined with the *P. aeruginosa* PAO1 fragment prey library constructed in the pUT18C vector, and the number of co-transformants obtained on MacConkey medium reached about 103 300 clones. Among the co-transformants, 33 were strongly red clones and the prey insert was further identified by DNA sequencing. In two clones out of 33 (~6 %), the prey fragment corresponded to the HsbRlib fragment of the RR HsbR, identical to the one obtained in the opposite screen (when HsbA was used as the bait) (Fig. 1). However, although we were able to prey HsbR when using HsbA as bait, the opposite approach using HsbR as the bait did not allow us to prey HsbA, mainly because *hsbA* combines a small size with an unpropitious *Sau*3A cleavage profile. Indeed, five *Sau*3A restriction sites are present in the 305 bp of the *hsbA* DNA sequence, and none of them is in-frame with the T18 subdomain borne by the pUT18C vector. To mitigate this bias, we also attempted additional screens using the pKT25-HsbRPP2C/ATPase bait construct combined with the *P. aeruginosa* PAO1 fragment prey libraries constructed in the pUT18, pUT18 + 1 and pUT18 + 2 vectors, and the number of co-transformants obtained on MacConkey medium reached approximately 71 000 clones each. However, this approach did not allow us to prey HsbA, probably due to the small coverage of these libraries.

All of the potential interactions observed in the different screens were further verified by reintroducing the bait and selected prey plasmids into BTH101 cells (Fig. 2a). Additionally, the coding sequences inserted in the bait and prey vectors were switched to test the interactions in the opposite orientation, and similar interactions were obtained (Fig. 2a). A red colour, indicating an interaction between preys and baits, was confirmed by a significant level of β-galactosidase activity in the corresponding clones as compared with paired controls (Fig. 2). The HsbRPP2C/ATPase-HsbA interaction confirmed results obtained previously (Bordi *et al.*, 2010; Hsu *et al.*, 2008). The D domain of the RR HsbR was found to dimerize (Fig. 2a), as previously demonstrated (Bordi *et al.*, 2010), and this is indeed a common feature of RR activation (Gao & Stock, 2010), which further leads to the activation of the output domain, which could be a DNA-binding or enzyme module.

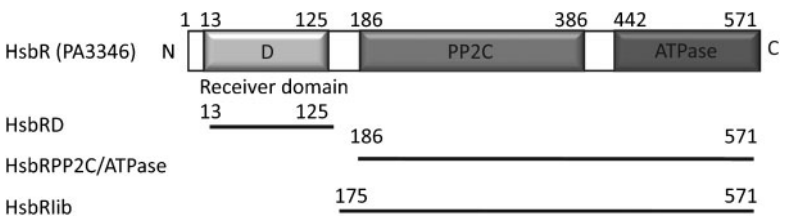

Fig. 1. Schematic representation of the RR HsbR (PA3346), a 571 aa protein formed of an N-terminal receiver domain (D) (amino acids 13–125) and a C-terminal effector domain (amino acids 186–571) containing PP2C (phosphatase C; amino acids 186–386) and predicted ATPase (amino acids 442–571) subdomains. Constructs (HsbRD, HsbRPP2C/ATPase and HsbRlib) made or obtained during library screening are also shown.

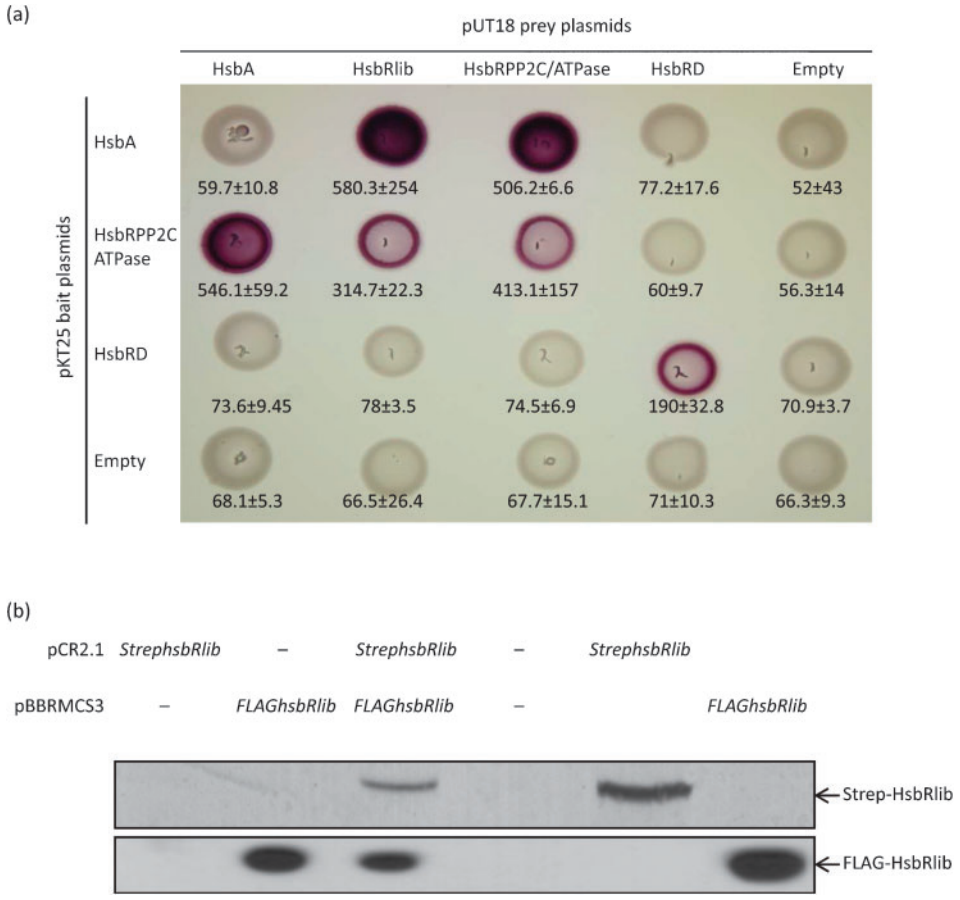

Fig. 2. (a) Double hybrid experiments. Interactions between HsbA and HsbR proteins or subdomains were tested using MacConkey medium and β-galactosidase assay independently of the cloning strategy in the bait and prey vectors. A red colony colour shows an interaction between proteins and was further confirmed by the β-galactosidase values obtained, while a white colour attests the absence of an interaction, a feature validated by weak or absent corresponding β-galactosidase values. (b) Pull-down experiments. N-terminal FLAG or Strep versions of HsbRlib were constructed in pBBRMCS3 and pCR2.1 vectors, respectively, and expressed in *E. coli*. Cell lysates were immunoprecipitated using anti-FLAG antibody-coupled beads, and FLAG-HsbRlib and Strep-HsbRlib derivatives were further detected using StrepTactin Alkaline Phosphatase conjugate and anti-FLAG antibody detection, respectively (first four lanes). Production of each version was checked separately (last two lanes).

Very interestingly, we additionally demonstrated that dimerization of HsbR could occur through its PP2C/ATPase effector domain. The dimerization of the PP2C/ATPase effector domain observed using our high-throughput *P. aeruginosa* two-hybrid bacterial library was further confirmed using pull-down experiments. Each N-terminal-tagged (FLAG or Strep) version of HsbRlib was efficiently produced (Fig. 2b). The Strep-HsbRlib product was pulled down with the FLAG-HsbRlib fragment immunoprecipitated with anti FLAG antibody-coupled beads, while appropriate controls were negative (Fig. 2b). The dimerization region of the HsbR effector domain is likely to be located in the PP2C/ATPase domain, as the interaction was also positive when we combined pKT25-HsbRPP2C/ATPase and pUT18C-HsbRPP2C/ATPase vectors (Fig. 2a). The dimerization of an effector domain is a very recent observation that has already been observed elsewhere. The structural characterization of WspR in *Pseudomonas syringae*, an RR with an effector domain that possesses diguanylate cyclase activity (GGDEF domain), showed that WspR is active as a tetramer (De *et al.*, 2009). This tetramer complex is a combination of two dimers of WspR (each dimer being formed by the interaction of two receiver domains of each WspR monomer) interacting via their GGDEF modules, and therefore the diguanylate cyclase activity of WspR relies on an oligomerization event that brings two GGDEF domains into close proximity (De *et al.*, 2009). No structure of a full-length RR with a PP2C/ATPase-type output domain has yet been solved, and it remains to be determined whether such a high-order oligomer could trigger PP2C/ATPase activity. However, the structural analysis of RsbU (Delumeau *et al.*, 2004), a protein which contains solely a PP2C domain involved in the positive activation of the general stress-response factor

σ^B in *Bacillus subtilis*, highlights that the RsbU phosphatase activity requires dimerization of its non-enzymic N-terminal domain. Taken together, these results confirmed the previous interaction demonstrated between the two proteins, HsbA, the putative anti anti-σ factor, and the RR HsbR, in particular through its PP2C/ATPase effector domain (Bordi *et al.*, 2010). These results also demonstrate the existence of a novel dimerization mechanism of the RR HsbR regulator through its own effector domain, which was further confirmed by pull-down experiments, highlighting the power of this approach. The effect of effector domain dimerization on HsbR function and activation remains to be elucidated.

Among the other identified preys obtained in the libraries that were capable of interacting with HsbR and HsbA were some interesting putative partners, whose involvement in the HptB/HsbR/HsbA signalling pathway requires extensive studies that are currently under way.

Conclusions

The genome-wide strategy using bacterial two-hybrid libraries presented here represents a powerful tool to identify unknown interactions, in particular in regulatory pathways, from which cognate partners are frequently dispersed throughout the genome. It allowed us to confirm the HsbA/HsbR interaction, and the dimerization of the RR HsbR through its receiver domain, but additionally and for the first time to observe dimerization of HsbR through its effector domain. This also paves the way for the identification of several interesting partners of the HptB/HsbR/HsbA pathway, which are under characterization. The genome-wide tools developed to decipher protein–protein interactions in the absence of any obvious candidate are well adapted to complex regulatory networks; however, this approach can be transposed easily to other physiological aspects of *P. aeruginosa*.

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