BACTOCOM

Bacterial Computing with Engineered Populations

Title: Distributed computable system by communicating sender strains with a transmitter strain by means of a conjugation device

WP4 - D 4.2 (due Month 36)

20 August 2013 / Version

Abstract

Deliverable D4.2 includes work corresponding to tasks 4.2, 4.3 and 4.4:

- Task 4.2. Summarize the properties of the propagation system, analyzed by monitoring transfer of AbR markers and other reporter systems.
- Task 4.3. Construct combinatorial plasmid series with engineered promoters and transcription factors.
- Task 4.4. Engineer a distributed computable system by communicating sender strains with a transmitter strain by means of a conjugation device.

Task 4.4 represents in fact the core of the Deliverable, since it deals with the construction and workings of the computable system. Task 4.2 in fact describes the implementation of the system. Task 4.3 was meant to produce the diversity of substrates on which the computable system will act. Therefore, we first describe the core of the system, we then describe its experimental implementation and, finally, we give some hints on the use of combinatorial plasmid

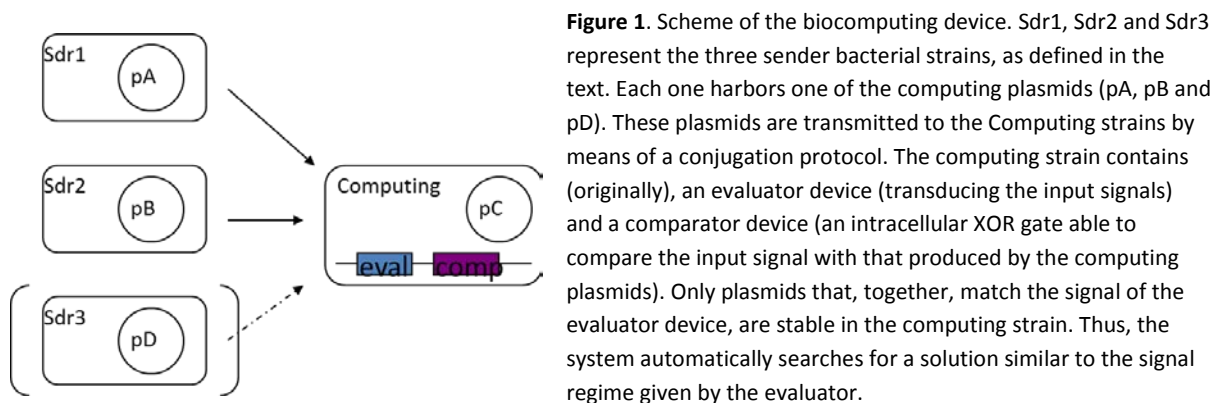|	
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communicating sender strains with a transmitter strain by
means of a conjugation device

System architecture

Figure 1 shows a scheme of the conjugation-based cellular computing system, which was constructed exactly as planned in the project Technical Annex.

Figure 1. Scheme of the biocomputing device. Sdr1, Sdr2 and Sdr3 represent the three sender bacterial strains, as defined in the text. Each one harbors one of the computing plasmids (pA, pB and pD). These plasmids are transmitted to the Computing strains by means of a conjugation protocol. The computing strain contains (originally), an evaluator device (transducing the input signals) and a comparator device (an intracellular XOR gate able to compare the input signal with that produced by the computing plasmids). Only plasmids that, together, match the signal of the evaluator device, are stable in the computing strain. Thus, the system automatically searches for a solution similar to the signal regime given by the evaluator.

In the final implementation of the system, each sender strain (Sdr) contained two plasmids: (i) a helper conjugative plasmid (a derivative of plasmid RP4) (Demarre et al., 2005) and (ii) one of the computing plasmid (either pA, pB or pD) containing an *oriT*(RP4), an AbR marker (ApR, CmR, etc.), a conditional replicator and expressing variants of a transcription factor controlled by a promoter from the library (see WP1). The replicator of the computing plasmids is under the control of Ptet promoter-operator. Compatible replicators originated from medium copy-number plasmids ColE1 (pA), pACYC184 (pB) and CloDF13 (pD) (Bartolome et al., 1991). In all cases, replication of the plasmid is controlled by an antisense RNA acting on the replication-primer promoter product, which is controlled by Ptet, analogously to the conditional plasmid replicators described by (Gil and Bouche, 1991). If a host strain expresses the *tet* repressor (TetR), the plasmid does not replicate and is lost. Plasmids pA, pB, etc. are stable in TetR-free strains such as the Sdr strains, but not in the computing strain if it is producing TetR.

The computing strain should contain an Evaluator device and a Comparator device, both inserted in the main *E. coli* chromosome. The output of the computing circuit is always protein CI, encoded by the cI lambda repressor gene. It acts on the Comparator together with the output of the Evaluator (which is the related repressor CI_{434}). If both repressors are expressed to similar levels (11 or 00 in a truth table), the Comparator produces no product. If they do not match (10 or 01), the comparator produces TetR, which impedes replication of the computing plasmids pA, pB, etc, and GFP, that can be quantified to follow the behavior of the system.

Alternatively, for the purposes of system testing, the computing strain contains a plasmid pC, which is a KmR, low-copy number plasmid based on the pSC101 replicon, which conditionally express the TetR repressor. Thus, pC can be used instead of the comparator in some specifically designed comparator-free tests.

System implementation

To fulfill the objectives of deliverable 4.2, we developed a high-throughput conjugation assay (HTCA) that allowed us to optimize conjugation rates in our experimental set-up for a series of conditions. These data is now available, to be used in WP5 to make a model of conjugation.

We developed a conjugation assay that can be monitored by cytometry, instead of the more laborious and time-consuming classical plate-out assay. Results obtained with both assay systems were compared. The conjugation assay was based on mobilizable plasmids containing R388 *oriT* and GFP under the control of the PtrwA promoter (one of the promoters in plasmid R388). In this system, expression of *trwA* is repressed in the donor, while GFP is induced upon transfer to a recipient strain (Figure 4.2). Thus, we can measure the kinetics of plasmid transfer of plasmids analogous to the conditional replicators shown in Deliverable 4.1.
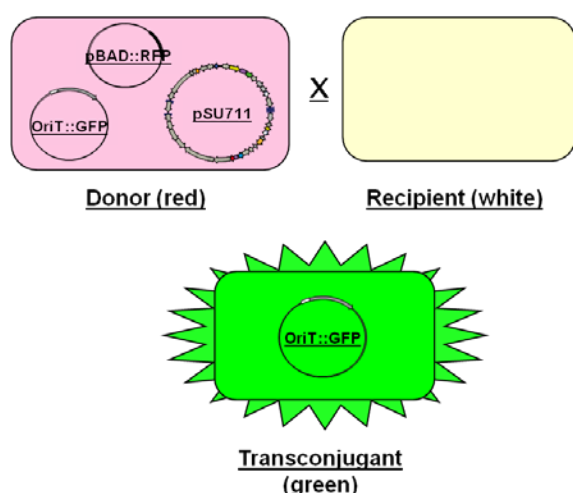

Figure 2. Scheme of the high throughput conjugation assay (HTCA). GFP synthesis is controlled by PtrwA promoter, which is repressed in the donor strain by the TrwA repressor, contained in the non-mobilizable helper plasmid pSU711. Upon conjugation, OriT::GFP enters the recipient strain, where GFP synthesis is not repressed, so it lights up.

Taking advantage of the HTCA, as well as standard plating-out assays, we determined the kinetics of conjugation or the reporter plasmids shown in Table 1, and optimized the system set-up to obtain almost 100% transfer within two transfer cycles. These results have been published (del Campo et al., 2012).

The intrinsic instability of vectors pB1.0 and pD1.0 (see Deliverable 4.1), as well as their incompatibility problems, were overcome in versions pB2.0 and pD2.0 by mutating the -10 box of promoter pTetA to the sequence of the -10 box of the native pRNAII promoter of plasmids pACYC184, CloDF13 and pBR322. These new variants were compatible among them and as stable as vector pA1.0 (Table 1). The estimated copy number of pA1.0, pB2.0 and pD2.0 were rather similar. Thus, they are more suitable for BACTOCOM purposes.

Table 1. Improved conditional-replication plasmids for bactocom cell-cell communication

Plasmid	Replicon	AbR	RNAII promoter	Conditional to	Copy number	% loss per generation
pA1.0	pBR322	Km	pTetA	TetR	470	<0.02
pB2.0	pACYC184	Cm	Hybrid	TetR	360	<0.02

			pTetA$_{-35}$ pRNAII$_{-10}$			
pE1.0 *	pBR322	Km	pLacZ	LacI	600	n.d.
pE2.0 *	pBR322	Km	pLacZ	LacI	n.d.	13.7
pE3.0 *	pBR322	Km	pLacZ	LacI	n.d.	0.97
pD2.0	CloDF13	Gm	Hybrid pTetA$_{-35}$ pRNAII$_{-10}$	TetR	370	<0.02

*pE1.0=pSU5046 in Table 4.1; pE2.0 contains tetR-LAA cloned under the promoter of trwA_R388 in the EcoRI/PstI sites of pE1.0 and mCherry in the PshAI site of pE1.0; pE3.0 is a pE2.0 derivative that contains gene *rop* under its natural promoter, cloned in the FseI site of pE2.0.
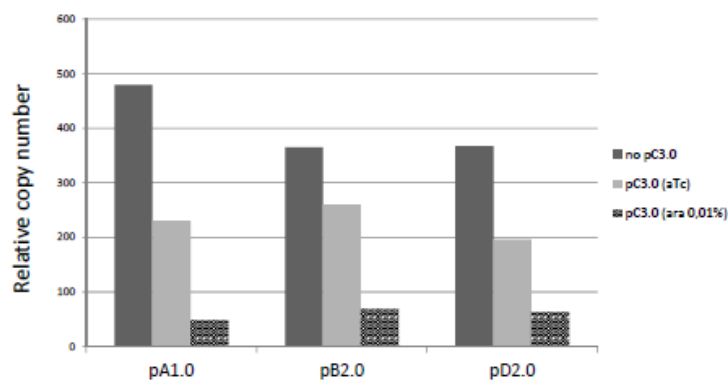

Figure 3. Copy numbers of conditional replication plasmids in the presence of different amounts of TetR. Dark grey bars represent copy numbers of the conditional vectors in the absence of TetR; light grey bars indicate copy numbers in the presence of repressor TetR-LAA + anhydrotetracycline, thus full activity of the repressor is prevented; dark dotted bars indicate copy numbers in the presence of higher amounts of repressor TetR-LAA, production of which is induced by arabinose. As can be observed, TetR-LAA acts by reducing copy numbers in all cases.
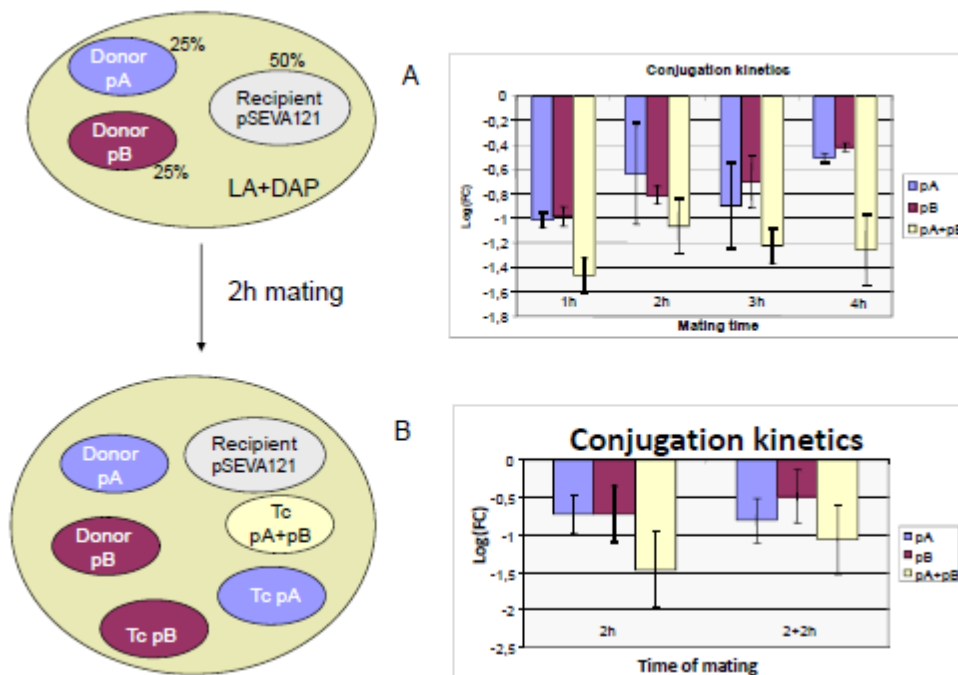
As a collateral project, we worked on an alternative protocol to stabilize ColE1-based replicons (such as the pBR322, pACYC184 and CloDF13 replicons used in bactocom). For this purpose, we analyzed the role of protein Rop in the stabilization of ColE1-like replicons by following the stability of vectors pE2.0 and pE3.0 by flow citometry. Both vectors are based on the pBR322 replicon but the production of *RNAII* is driven from pLac promoter. It is known that Rop stabilizes the pair *RNAII-RNAI*, decreasing the average plasmid copy number but also constricting the copy number deviation. As shown in Table 1, when gene *rop* was encoded in *cis,* the stability of the vector dramatically increased. Other experiments, not shown in Table 1, suggest that vector stability does not increase when Rop is supplied in trans (implying that a *rop* gene has to be added to each relevant replicon to increase its stability). This result is significant for bactocom since we have observed that altering the cargo genes in any ColE1-based vector might alter its stability and incompatibility properties. Addition of *rop* might alleviate these problems.

Two sender populations, each containing a conditional vector tagged with a fluorescence marker (cerulean fluorescence protein in case of pA1.0 and mcherry for pB2.0)) were mixed with a computing strain (*E. coli* BW 27783) that contained the TetR-producing vector in several mating experiments. The final aim of these experiments was to provide data our modeling team to optimize in silico the experimental parameters needed to optimize the ratios of pA, pB and pC in a given bactocom experiment without needing to test them experimentally.

To mobilize the conditional vectors (based on *oriT*_RP4) by conjugation, the donor must contain the conjugative genes of RP4. Thus, several variants of donor strains were tested: i) DH5α containing RP4

as an autonomous plasmid, ii) S17.1, and iii) MFDPir. The last two contained RP4 integrated in the chromosome. Option i) was ruled out due to entry exclusion problems that diminished the production of double transconjugants containing both pA and pB. Option ii) was ruled out due to problems associated with strain S17.1, such as Hfr, excision and transfer of RP4 and phage Mu, after repeated testing. We finally chose the third option, in which the sender strain lacks phage Mu and the *nic* site of RP4, thus providing the functions needed for transferring the mobilizable vectors, while avoiding the inconveniences of an Hfr strain.

A first set of mating experiments was carried out to define the transfer kinetics of the conditional vectors to a recipient strain devoid of TetR as a control (plasmid pSEVA121, an RP4 replicon compatible with pA and pB, in which TetR-LAA was cloned under promoter P_{BAD} and the regulation of *araC*, given pC). Different conjugation times (shown in panel A of Figure 4) and successive rounds of mating transferring the conjugative mix to fresh media (panel B of Figure 4) were tested. As seen in Figure 4 panel A, individual conjugation frequencies (for pA or pB) progressively increase with time. Nevertheless, double transconjugants (pA + pB) barely increase after 2h. Two rounds of 2h matings were found to slightly increase the frequency of double transconjugants, in accordance to previous results (del Campo et al., 2012).

Figure 4. Conjugation kinetics of plasmids pA and pB. Left: a scheme of the conjugation process. The initial bacterial population contains donor cells containing either pA or pB plus recipient cells containing pSEVA121. After a given mating time, the population contains those three types for bacteria plus three others, namely transconjugant cells (Tc) containing either pA, pB or both. Right: Kinetics of pA and pB conjugation. Panels A and B show the conjugation frequencies of both plasmids after different mating times. In (A), the mixture was set up with the proportions shown at the left and conjugation allowed to proceed for different times (1 to 4h). In (B), the population of the mating plate was mixed after 2h and allowed to mate for an additional 2h period. All experiments were carried out at 37ºC. Conjugation frequencies are represented as the Log_{10} of the ratio of transconjugants per recipient cell (T/R+T). The average and standard deviation of at least three experiments, each performed by duplicate, are shown in the figures.

The results in Figure 4 are not entirely satisfactory, since the ratio of double transconjugants (pA + pB) was at the most 10% of the total recipient population, when we aimed at 100% double transconjugants. The reasons why infection of recipient populations by conjugative plasmids reaches a plateau before full infection are obscure. We checked for the doubling time of each strain used in the mating experiments as a possible explanation. Results are shown in Figure 5.
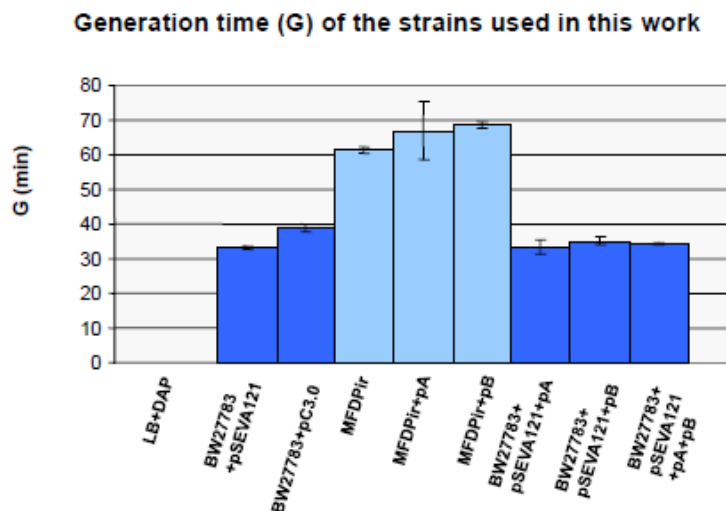

Figure 5. Generation times of strains used in this work. For each strain, 12 replicas were grown in separate wells of a 96-well plate, using a 1/10.000 dilution of an o/n culture. Each well contained 150 µL LB + 0.3 mM DAP. Plates were incubated at 37ºC in a Victor-3 fluorimeter, and OD_{600} measures were taken each 5 min for a total of 14h. The steep of the corresponding lines were obtained from the Log readings of each well. The generation times (G, in minutes), were calculated following the equation: G = ln2/steep of the line.

Regardless the presence of the conditional vectors, donor strain MFDPir showed longer generation times than the recipient strain BW27783, as well as the single and double transconjugants. Thus, it is likely that both recipients and transconjugants largely overgrow donors on the mating surface. This might explain why we see little improvement by repeated mating (probably donors have almost disappeared from the population after a few hours of mixed growth). A possible solution could be to add more donors for the second (and successive) round of conjugation.

The following set of mating experiments is shown in Figure 6. They were carried out using the 2h+2h set up (the same conditions as in Figure 4 panel B). In this case, the recipient strain did contain a vector pC encoding repressor TetR-LAA, which impedes replication of the conditional vectors pA and pB. The LAA version of this repressor has a shorter half-life than TetR, allowing longer residence times of pA and pB in the recipient (computing) strain, depending on the amount of repressor, which is controlled in turn by the amount of arabinose (the inducer of promoter P_{BAD} that controls TetR-LAA production).
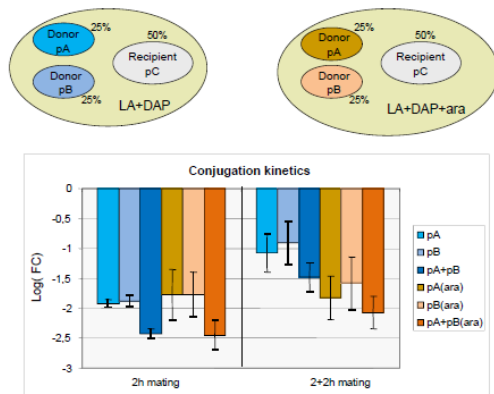
Figure 6. Conjugation kinetics of pA and pB plasmids to recipient cells in the presence of plasmid pC (producing TetR). See Figure 4 for details.

As shown in the figure, the presence of arabinose in the mating media (i.e., large amount of TetR) reduced the apparent conjugation frequency of both single and double transconjugants as compared to absence of arabinose (when only basal amounts of TetR-LAA are produced). Since pC does not reduce conjugation efficiencies, these effects must be produced by the increased instability of plasmids pA and pB in the recipient strain.

In conclusion, under the conditions tested, we achieved a maximum of 0.1 double transconjugants/recipient cell in the absence of pC, which was reduced to 0.03 double transconjugants/recipient cell when pC (producing TetR-LAA at basal level) was present and dropped down to 0.01 double transconjugants/recipient cell when TetR-LAA was overexpressed by the presence of arabinose. The datasets obtained in these experiments can be used in WP5 to develop models that simulate bactocom experiments.

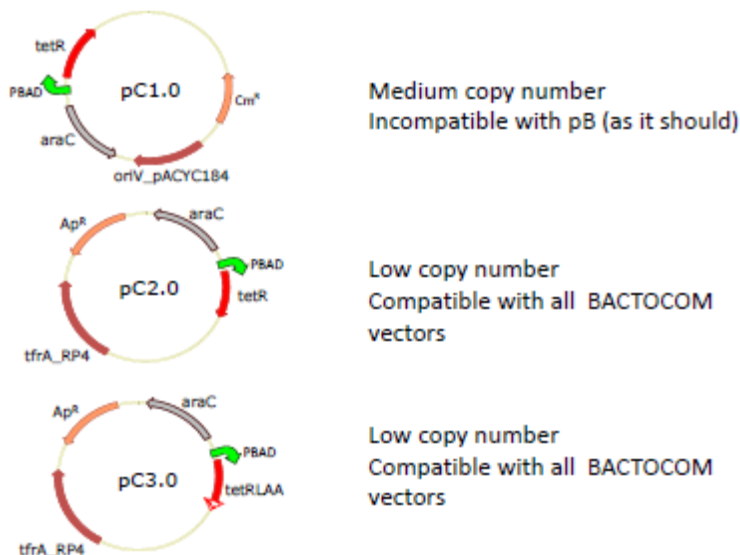Three variants of the computing plasmid pC were constructed (Figure 7).

Figure 7. Relevant properties of variant pC plasmids constructed for this work.

After testing the stability of the conditional vectors with these pC variants, we finally chose pC3.0, which encodes the unstable variant TetR-LAA, which showed best compatibility with the conditional vectors (pC2.0 acted too rapidly). Plasmid pC3.0 allowed us to perform a comparator-independent computing experiment in which both conditional replicators compete in the presence of increasing amounts of TetR-LAA, as illustrated in Figure 8. Fine-tuning of pTetA promoter in vectors pB and pD, as well as the construction of the unstable TetR variant TetR-LAA were necessary to overcome

previous problems found in the implementation of the experimental bactocom setting. Now the system, as planned originally, can be used to test for conditions that produce TetR, in which both plasmids are lost, or do not produce TetR, in which both plasmids are stably inherited.
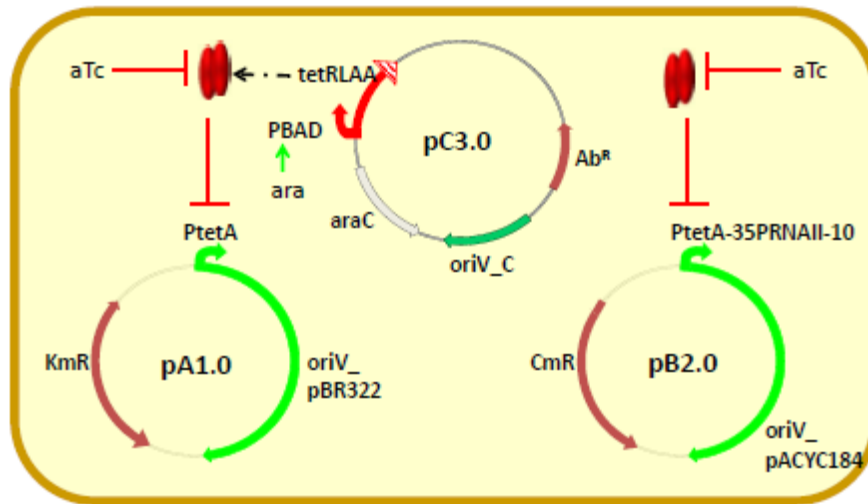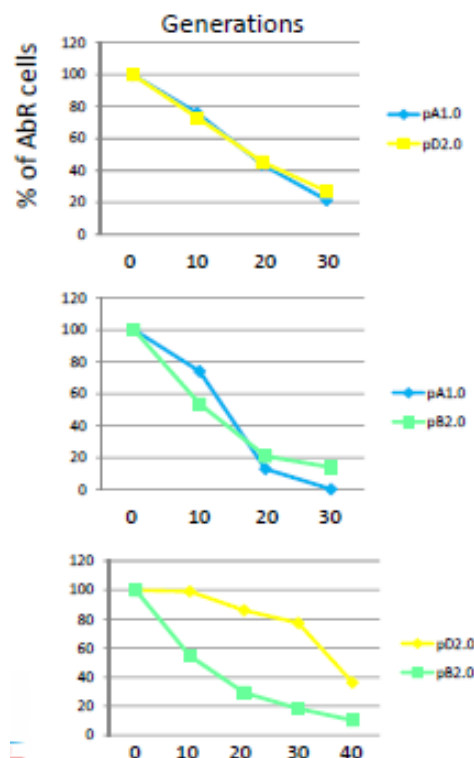

Figure 8. Scheme of a three-plasmid strain to test for conditional replication of the test plasmids.

E. coli strain BW27783 was used to test all pairs of conditional replicators (pA1.0+pB2.0, pA1.0+pD2.0, pB2.0+pD2.0) in the presence of pC3.0 (using arabinose 0.001%). The stability behavior of these combinations is shown in **Figure 9** (left).

Pairs pA1.0+pB2.0 and pA1.0+pD2.0 showed the best behavior because each vector of the pair is lost at a comparable rate in the presence of TetR-LAA. Those plasmid combinations were completely stable in the absence of TetR-LAA and only slightly unstable (<2% / 10 generations) in the presence of basal levels of TetR-LAA.

Combinatorial plasmid series with engineered promoters and transcription factors

In order to accomplish this objective, we concentrated in setting up a bactocom-based comparator-free strategy for automatic optimization of switchable regulators of the MerR family. MerR regulators are orthogonal to *E. coli* (they are typically based on mobile genetic elements, and the *E. coli* chromosome contains no close homologs of them), they have dual activity, acting both as

repressors and activators, and they respond to different metal ions, for instance, Hg^{2+} (Brown et al., 2003). Figure 10 shows the typical response of the Pmer promoter, in the presence of MerR depending on the presence of the inducer ($HgCl_2$). As can be seen in the figure, Pmer is an intrinsically weak promoter, giving about 900 arbitrary fluorescence units (AFU). MerR acts as a repressor in the absence of inducer, reducing Pmer expression to background levels (about 100 arbitrary AFU). Conversely, addition of 0.5 µM $HgCl_2$ leads to an increase in expression, to about 8,000 AFU. Thus, MerR acts as a repressor or an activator, depending on the inducer Hg^{2+}.
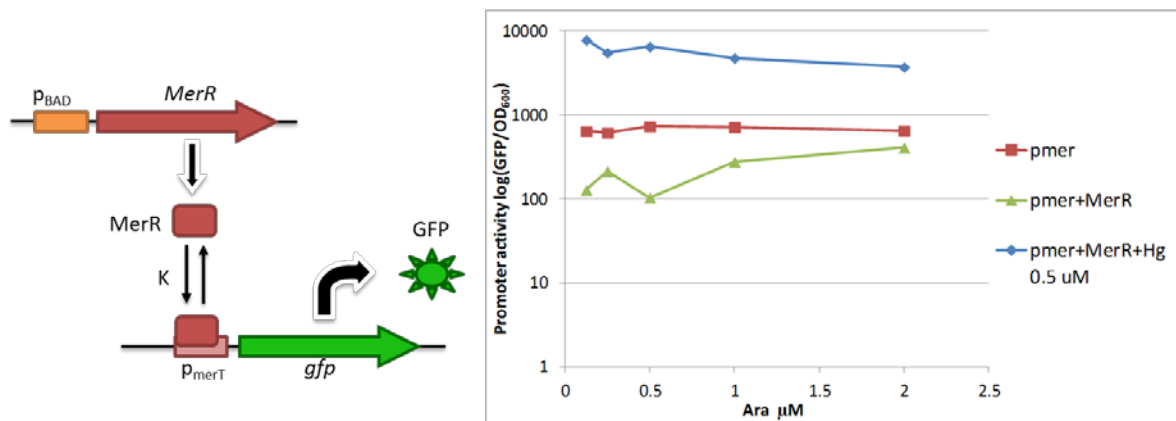

Figure 10. Response of the Pmer promoter. The drawing at the left shows the scheme of the experimental setup, in which MerR expression is under the control of Pbad (the arabinose promoter), and Pmer promoter controls the expression of GFP. The graph at the right shows the expression levels of Pmer (as reflected by the level of GFP activity) under different expression conditions, as represented by the codes shown at the right of the graph.

We decided to attack this task in increasingly complex set-ups, as shown below. First we decided to optimize a promoter and a regulator responsive to mercury, using the two-plasmid system represented in Figure 11, which just requires plasmids pA and pC. In this set-up we can optimize MerR/Pmer variants in which Pmer is repressible by MerR in the presence of Hg2+. We will later use a three plasmid system, as represented in Figure 12, in which MerR will act as an activator in the presence of Hg. If our system is successful, the same set-up will be used to optimize other metal-sensors (Cd, Sr, etc). These experiments are in progress and should produce a series of metal sensors and other modulatory devices that will be generally useful in synthetic biology projects.

Two-plasmid experiment: Optimization of a Pmer / MerR pair in which MerR acts as a strong repressor of a strong promoter. The experimental setup is shown in Figure 11. Plasmid pA enters the computing strain by conjugation, since it contains *oriT*_R388. pC1::Pmer-GFP on its own produces little GFP (maybe not detectable) but enough TetR to make pA unstable. In a strain containing pA::Para-MerR + pC1::Pmer-GFP), two scenarios are possible, depending of the presence/absence of Hg+2:

➢ In the absence of Hg2+, MerR is a repressor. Thus, pA should be stable. If we use libraries of MerR and Pmer mutants, the system will automatically select for the best repressible pairs. We can select for KmR (or Km + Ap) to follow the selection process.

➢ In the presence of Hg2+, wild type MerR is an activator, so pA should be lost. Pbad-MerR + pC should express GFP and display green colonies. On the other hand, if a given MerR, acting on a variant of Pmer, does not activate the promoter, but enhances its repressor activity, that will result in a Pmer/MerR pair that will act as a Hg2+-inducible repressor. This device

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does not yet exist in nature. Its construction will pave the way for the construction of many similar devices.
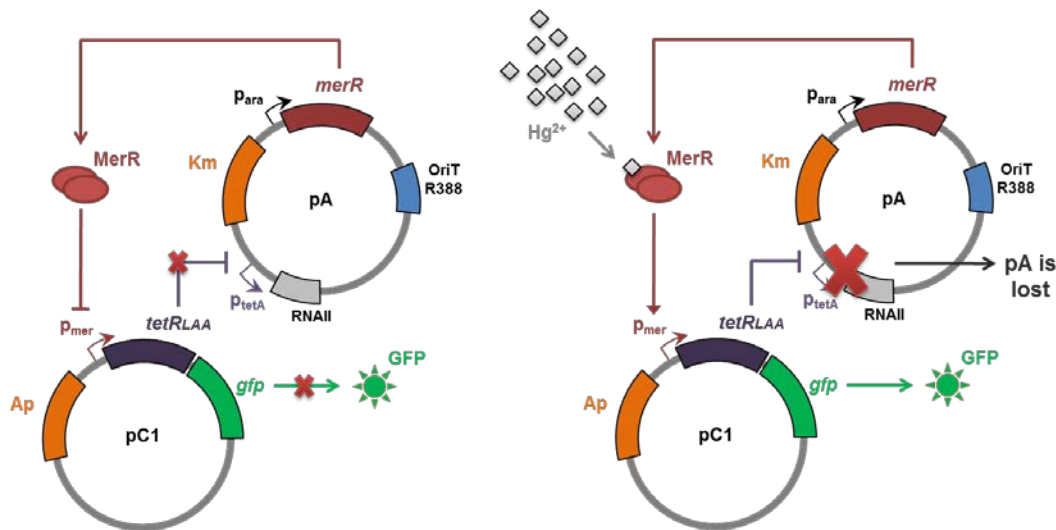

Figure 11. Two-plasmid experiment for the automatic selection of MerR /Pmer combinations that act as Hg-dependent repressors. The drawings represent the behavior of the wild-type situation. The objective of the experiment is to select a Pmer/MerR pair that acts as a Hg-dependent repressor instead of as an activator. See text for details.

Three-plasmid experiment: Optimization of a Pmer / MerR pair in which MerR acts as a strong activator of a very weak promoter. The experimental setup is shown in Figure 12. In this case, pC2 contains pR_lambda instead of Pmer. pA contains *oriT*_R388 in order to mobilize it to the computing strain, which contains pB and pC2. Before pA arrival, cI repressor is not produced in a concentration high enough to inhibit P_R-controlled TetR expression. When pA enters the computing cell, two scenarios are possible, depending of the presence/absence of Hg^{2+}:

➢ In the absence of Hg^{2+}, MerR is a repressor. Thus, pA should be even more unstable. If we use libraries of MerR and Pmer mutants, the system will automatically select for worse repressible pairs.

➢ In the presence of Hg^{2+}, wild type MerR is an activator, so cI production will be induced and repression of P_R will lead to less TetR and pA should be stable. The stronger the activation, the more potent selection will be operating on pA replication. Thus, the system will automatically select variants of MerR/Pmer which are strong activators. This device improves those which exist in nature (since MerR is not a strong activator when acting on the wt Pmerpromoter). The construction of such a device will pave the way for the construction of many similar devices, using other metals, etc.
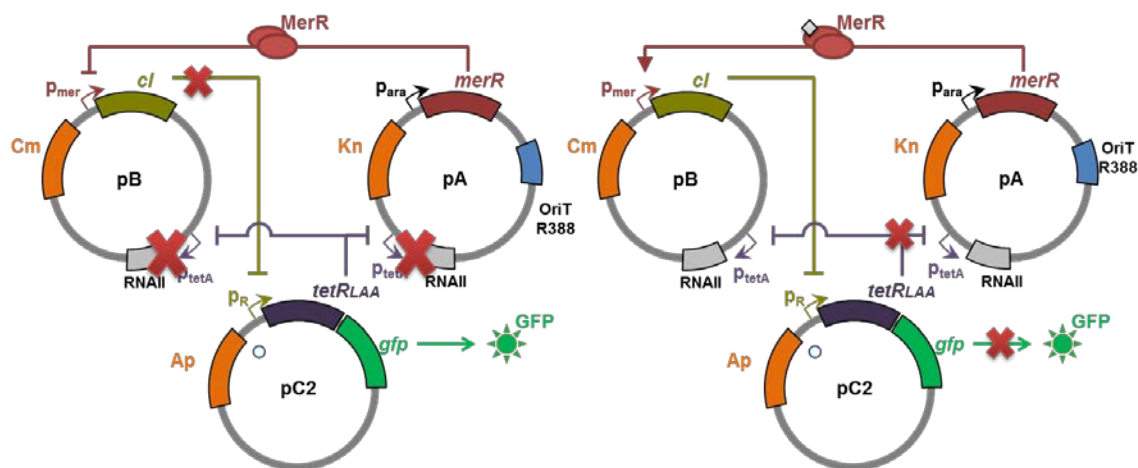
Figure 12. Three-plasmid experiment for the automatic selection of MerR /Pmer combinations that act as Hg-dependent activators. The drawings represent the behavior of the wild-type situation. Plasmid pA enters the computing strain by conjugation. The objective of the experiment is to select a Pmer/MerR pair that acts as a Hg-dependent activator (stronger than wild-type). See text for details.

At the time of writing this deliverable, we are still implementing and testing these experiments. Unfortunately, no sufficient results have been obtained yet to be incorporated here. We hope to be able to show significant advances in the next few weeks (in time for the final review).

Perspectives

BACTOCOM uses a conjugation protocol to assemble together, within the same computing cells, two or three plasmids that will test a given genetic circuit and automatically find a solution. When a given computing cell finds a solution, the plasmids contained in it will be stable, or at least more stable than in those containing bad solutions to the problem. A bottleneck of BACTOCOM is therefore how rapidly the population selects the solution to the problem, which will obviously depend on its complexity. In order to accelerate the selection process, it would be desirable that the good solutions could proliferate differentially. This can be achieved by second-round conjugation if the plasmids involved were not mere *oriTs* but fully functional conjugative plasmids. We demonstrate in deliverable 4.3 that newly formed transconjugants are better donors than the donors themselves. In deliverable 4.2 we describe the basic functioning of the system.

The plasmids constructed for this deliverable, and the experimental implementation described here form the core of BACTOCOM biocomputing device. We demonstrate that using conjugation as a cell-cell communication protocol, we can efficiently deliver two or three plasmids to a given computing cell. We further demonstrate that the automatic selection protocol works efficiently and should be able to find solutions in a suitable comparator device is available. Finally, we test the system in a comparator-free scenario and try to automatically select improved Hg-sensing devices.

Sharing and distribution

Plasmids already available in our bactocom-DB, and those that will enrich the collection in the future, will be distributed world-wide to researchers at any academic or non-profit laboratory. No MTAs will be requested prior to shipping the constructs to potential users of such categories. In case of utilization for commercial or industrial purposes, solicitors should be aware that some of the DNA sequences and/or the bactocom-constructed plasmids themselves could be subject to intellectual property restrictions, for which we disclaim any carrier liability. Clones will be distributed either as glycerol stocks, stabs in agar tubes or purified plasmid DNA (when appropriate).

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