EvoEvo Deliverable 1.1

Dominique Schneider, Guillaume Beslon, Santiago F. Elena, Otmane Lamrabet

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TEV and *E. coli* strains for robustness analysis

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Person in charge: Dominique Schneider

Partner in charge: UJF

Workpackage: WP1 (Experimental observation of EvoEvo in action)

Deliverable description: TEV and *E. coli* strains for robustness analysis: Production of TEV populations with diverse degrees of mutational robustness and of different genomic architectures; production of *crp*-deleted bacterial strains and identification of combinations strains-environments affecting bacterial robustness.

Revisions:

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1.1	Adding sections on TEV	18/11/14	S. Elena (CSIC)
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1.7	Corrections and validation by D. Schneider and S. Elena	16/12/14	D. Schneider (UJF)

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1. Introduction

Workpackage 1 of the EvoEvo project aims at observing and quantifying experimentally the "EvoEvo strategies" of microorganisms and relate it to their robustness, evolvability and open-endedness. Two complementary micro-organisms will be studied in this workpackage: a virus (*Tobacco etch virus* – TEV) and a bacterium (*Escherichia coli*). The evolutionary properties of these two organisms will be studied using *in vivo* experimental evolution. More precisely, we will propagate living organisms for hundreds to tens of thousands of generations in defined environments [Hindré *et al*., 2012]. Both models used in this project are classic models in experimental evolution that allow detailed genetic manipulations and analyses. Furthermore, given their short generation times, large population sizes and, in the case of the RNA virus high mutation rates, relevant evolutionary changes take place after short periods of time, allowing us to observe evolution in action.

To observe EvoEvo strategies in the two model organisms, we will use the following strategy: by engineering the genomes (and in some experiments the environment) of the organisms in different ways, we will create evolutionary perturbations and observe how the organisms evolution will cope with these perturbations in terms of robustness (whether the perturbation in harmful or not), evolvability (whether organisms carrying the perturbation are able to recover or not) and open-endedness (whether the organisms carrying – or not – the perturbation are able to adapt to new environments).

The first step of this strategy is to produce the engineered genomes in both models. This document presents the work conducted by partner 2 (UJF) and partner 5 (CSIC) to this aim as well as the results obtained so far (produces strains and their first analysis[1]). It is organized in two sections (one per model organism) since the engineering methodologies are different in bacteria and viruses. Each section starts with a short introduction to the considered model.

2. Task 1.1, Section 1: Robustness at the population level in the TEV experimental model

Tobacco etch virus (TEV; genus *Potyvirus*, family *Potyviridae*) is a plant virus infecting hundreds of crops and wild plants though most of their natural hosts belong to the economically important *Solanaceae* family (*e.g*., tomatoes, potatoes, tobacco…). TEV consists of a genomic RNA strand of ~9.5 Kb linked at the 5' end to a viral protein (VPg) and with a poly(A) tail at the 3' end. It encodes eleven mature gene products that result from the processing of a large polyprotein by three viral proteases, and a second polypeptide derived from a translational read-through process (relevant for the present study are the *NIb* cistron that encodes the viral RNA-dependent RNA polymerase, the *CP* cistron encoding the coat protein, the *NIaPro* cistron encoding the main viral proteinase involved in the proteolytic maturation of the polyprotein, and the *HC-Pro* cistron that

[1] Note that, formally, deliverable D1.1 is composed of the engineered strains, all of them being available on request in UJF (*E. coli* strains) and CSIC (TEV strains). The present document summarizes the work done and describes the strains produced with their ID (see Table 1 pages 5 and 6 for the TEV strains, and Table 3 pages 10, 11 and 12 for the *E. coli* strains). As such, it does not constitute the deliverable *per se*.

encodes a multifunctional protein involved in aphid transmission, and a proteinase involved in genome amplification and suppression of plant RNA silencing defense).

As a consequence of high mutation rates, natural selection may have favored the evolution of genomic robustness mechanisms in riboviruses. These mechanisms can be either intrinsic (inherent to the genetic architecture and replication mode) or extrinsic (the result of interacting with cellular factors). Previous results suggest that RNA viruses adopt an anti-robustness strategy based on individual hypersensitivity to mutations that allows the average population fitness to be maintained high due to the efficiency of selection in purging deleterious genomes from the population. Whether mutational robustness can evolve in such conditions is an open question. It has been theoretically established under which demographic and mutational conditions individual genomes would evolve robustness or hypersensitivity (i.e., population robustness). By simulating these conditions in a context of experimental evolution, would it be possible to alter TEV hypersensitivity to point mutations?

In deliverable D1.1, we have generated a collection of TEV strains and evolved populations that differ in their gene order and content, some contain additional genes (increased complexity), some contain fewer genes (reduced complexity), some contain multiple copies of a gene (functional and genetic redundancy), and some contain additional genes of redundant function (functional redundancy). All these genotypes have been (or are still being) evolved under the conditions described in the previous paragraph.

2.1. Production of TEV strains and populations with diverse degrees of mutational robustness and of different genomic architectures

2.1.1. Genetic engineering of TEV genomes

During this first year, we have been performing intense genetic engineering to generate 10 new TEV strains (Table 1). These strains can broadly classify into four categories: genotypes with genetic and functional redundancy (expected to be more robust against mutation), genotypes with simplified genomes (expected to be more sensitive against mutation), genotypes with new acquired functions (expected to be more evolvable), and genotypes with altered gene orders (also expected to be more evolvable). Among the first group, we proceeded to duplicate essential viral genes. We have created genotypes with two copies of the replicase cistron *NIb*, two copies of the CP cistron encoding for the coat protein, two copies of the *NIaPro* cistron encoding of the major protease, and two copies of the multifunctional protein helper-component proteinase silencing-suppressor encoded by the HC-Pro cistron. For the second group, we created a genotype lacking an essential viral gene, the NIb replicase. This genotype is only viable in *Nicotiana tabacum* plants that express NIb protein transgenically (*Nt*::*NIb* plants). For the third group, we created genotypes carrying useless new functions (*e.g.*, the green fluorescent protein eGFP), genotypes carrying functional genes, such as a second suppressor of RNA silencing (*e.g.*, the cucumovirus 2b protein; functional redundancy but not genetic redundancy) or a host gene involved in modification of the methylation status of RNA and DNA (e.g., *alkB*). Finally, for the fourth group, we have permutated the position of *NIb* to all possible proteolytic sites in TEV polyprotein.

All constructs were made using the pMTEV infectious cDNA plasmid and are maintained in electro-transformed *Escherichia coli* strain DH5α. For the double *NIb* and *NIaPro* constructs, we tried two different cloning sites. In these plasmids, infectious vRNA can be generated by *in vitro* transcription from the T7 promoter cloned upstream of the +1 nucleotide of TEV genome. The viability of the resulting genotypes was tested by inoculation of susceptible *N. tabacum* var Xanthi

plants with a fix amount of in vitro transcribed vRNA. Table 1 summarizes the viability of each construct as well as their current evolution status.

Table 1: List of TEV strains generated for WP1 and their evolution status.

Clone	Insertion site	Viability plasmid	Viability virus *in vivo*	Symptoms	Status
TEV-2*NIb*1	5'UTR/*P1*	Yes	Yes	Normal	Evolved, molecular analyses in progress; to evaluate robustness
TEV-2*NIb*2	*P1*/*HC-Pro*	Yes	Yes	Normal	Evolved, molecular analyses in progress; to evaluate robustness
TEV-2*CP*	*NIb*/*CP*	Yes	Unstable after 1 passage	Normal	Discarded
TEV-2*NIaPro*1	*P1*/*HC-Pro*	Yes	Yes	Weaker, slow progression	Evolving; to evaluate robustness
TEV-2*NIaPro*2	*NIb*/*CP*	Yes	Yes	Weaker, slow progression	Evolving; to evaluate robustness
TEV-*2HC-Pro*	*P1*/*HC-Pro*	Yes	Yes	Normal	Evolving; to evaluate robustness
TEV-Δ*NIb*		Yes	Yes (in *Nt*::*NIb* plants)	Normal	Evolved; to evaluate robustness
TEV-eGFP	*P1*/*HC-Pro*	Yes	Yes	Normal	Evolved; to evaluate robustness
TEV-*2b*	*P1*/*HC-Pro*	Yes	Yes	Stronger	Evolving; to evaluate robustness
TEV-*alkB*	*P1*/*HC-Pro*	Yes	Yes	Weaker, slow progression	Evolving; to evaluate robustness
TEV-*NIb*1	5'UTR/*P1*	Yes	Yes	Weaker, slow progression	Evolved; molecular analyses in progress; to evaluate robustness
TEV-*NIb*2	*P1*/*HC-Pro*	Yes	Yes	Weaker, slow progression	Evolved; molecular analyses in progress; to evaluate robustness

TEV-*NIb*3	*HC-Pro*/*P3*	Yes	Yes (in *Nt*::*NIb* plants)	Normal	Discarded
TEV-*NIb*4	*P3*/*6k1*	Yes	Non-viable		Discarded
TEV-*NIb*5	*6k1*/*CI*	Yes	Non-viable		Discarded
TEV-*NIb*6	*CI*/*6k2*	Yes	Non-viable		Discarded
TEV-*NIb*7	*6k2*/*VPg*	Yes	Non-viable		Discarded
TEV-*NIb*8	*VPg*/*NIaPro*	Yes	Non-viable		Discarded
TEV-*NIb*9	*CP*/3'UTR	Yes	Non-viable		Discarded

2.1.2. Experimental evolution of engineered TEV strains

The designed evolution experiments sought to test the hypothesis that RNA Viruses adopted an anti-robustness strategy based on individual hypersensitivity to mutations that allows the average population fitness to be maintained high due to the efficiency of selection in purging deleterious genomes from the population. In theory, such strategy evolves in very large populations submitted to high mutation rate [Krakauer & Plotkin, 2002]. By contrast, evolving at small populations and low mutation rates may favor alternative robustness strategies based on individual robustness. To test these hypotheses, we have created contrasting experimental conditions in which effective population size (N_e) and genomic mutation rate (U) are manipulated. Table 2 shows the four experimental conditions in which evolution experiments are taking place. U can easily be manipulated by treating the vRNA with the appropriated amount to the chemical mutagen HNO_2. N_e = 1 can easily by obtained by inoculation in the local lesion host *Chenopodium quinoa* and isolation of a single individual local lesion (i.e., generated by a single viral particle). N_e = 10^4 are attained by serial dilution-titration of the infectious sap generated from infected plants at the previous evolutionary passage during. Ten independently lineages have been (or are being) evolved at each of the four experimental conditions. Evolution phase will expand for 25 serial passages in the natural host *N. tabacum*.

Table 2: Experimental design for robustness/anti-robustness experiment

	Genomic mutation rate (U)	
Effective population size (N_e)	**Standard (U = 0.5)**	**Increased (U = 5)**
Small (N_e = 1)	Expected: evolution of more robust individuals	Expected: extinction by mutational meltdown
Large (N_e = 10^4)	Expected: no change from the background condition	Expected: evolution of more brittle individuals

As indicated in Table 1, the evolution phase has already been finished for six TEV strains (indicated in green in Table 1), and we have thus created 40 × 6 = 60 populations. We are now in engaged in the process of evolving five other strains (they are actually at different intermediate

passages of evolution; indicated in orange in Table 1). Eight more strains have been proved to be non viable in *N. tabacum* and thus are not being evolved.

2.1.3. Setting up an experimental system to quantify mutational robustness

Essential for this project is the ability to experimentally evaluate mutational robustness in an easy yet highly reproducible manner. To do so, we have been developing a method based in chemical mutagenesis and evaluation of the infectivity of mutagenized genomes. In short, an *in vitro* mutagenesis procedure HNO_2 was optimized for TEV sap. The more robust a genotype, the less affected is expected to be to incubation with HNO_2. Infected tissue was collected and analyzed after 5, 6 and 7 dpi. Fig. 1 summarizes the results of the method for the wild type TEV strain used to set up the technique. The two left panels show the results for symptoms severity (in a semi-quantitative scale of 4 categories), the two right panels show the results for the infectivity (percentage of infected plants inoculated with a given amount of vRNA). The two upper panels were generated for a wider incubation period. The two lower for a narrower one. Optimal results are for incubation periods of 3, 3.5 and 4 hours.

To confirm the amount of mutations introduced by the mutagen at different incubation times, we are using Illumina NGS. Results should be sent back by the subcontracted company soon.

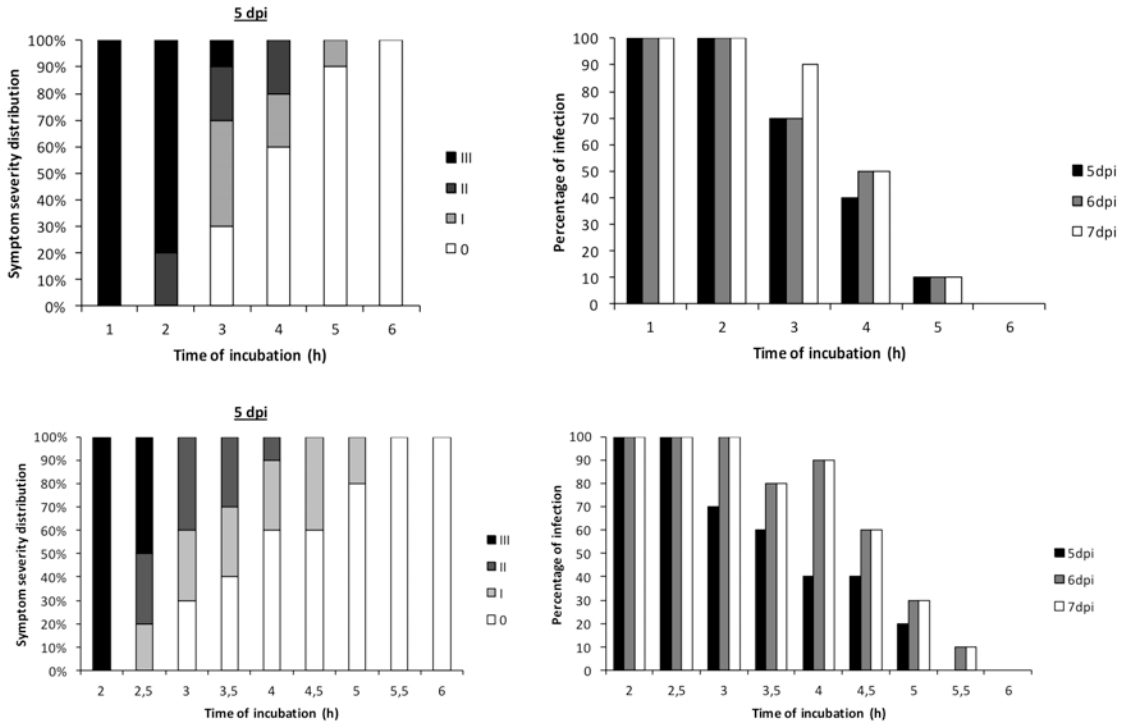

Figure 1. Results of the HNO mutagenesis for wild type TEV.

2.2. Articles published within the framework of this study

Tromas, N., Zwart, M.P., Forment, J., Elena, S.F. (2014). Shrinkage of genome size in a plant RNA virus upon transfer of an essential viral gene into the host genome. *Genome Biol. Evol*. **6**(3): 538-550, doi: 10.1093/gbe/evu036.

3. Task 1.1, section 2: Robustness at the regulatory network level in the *E. coli* experimental model

During a long-term evolution experiment (LTEE), twelve populations are propagated by daily transfers from a common *Escherichia coli* ancestor for more than 60,000 generations in a constant glucose-limited environment. Adaptation of *E. coli* during the LTEE has been shown to involve the rewiring of global regulatory networks [Hindré et al., 2012]. In particular, the CRP-controlled regulon has been shown to be increasingly important during evolution [Cooper et al., 2008]. Deletions of the global regulatory *crp* gene have been introduced in both the ancestor of the LTEE and two independently evolved clones, one sampled from each of two of the twelve populations after 20,000 generations [Cooper et al., 2008]. Deleting *crp* had a much more dramatic effect on both the growth in the evolution environment and the global transcription profile of the two evolved clones than of the ancestor. Because the sequence of the *crp* gene was unchanged after 20,000 generations during evolution, these differences indicated epistatic interactions between *crp* and mutations at other loci that accumulated during evolution [Cooper et al., 2008]. Therefore, epistasis has been important in the adaptive evolution of these bacterial populations. Identification of the types of genetic changes that epistatically interact with the *crp* deletion would give new insights into the mechanisms through which epistasis can evolve. Moreover, phenotypic investigation of these changes in the global regulatory network will allow us to address whether these modifications of the interactions between global regulators within the regulatory network affected the robustness of the evolved clones. However, the *crp* deletions have been constructed in evolved clones from only two of the twelve populations. Therefore, we decided to construct *crp* deletions in all twelve populations.

This deliverable included the construction of the *crp* deletions in evolved strains from all twelve populations and the investigation of the effect of these deletions in different environments, including the evolution environment and alternative ones. We will then be able to address whether deleting *crp* in the ancestor and after evolution resulted in differential growth defects. Therefore, we can investigate the impact of rewiring regulatory network on robustness.

3.1. Production of *crp*-deleted bacterial strains and identification of combinations strains-environments affecting bacterial robustness

3.1.1. *crp*-deleted bacterial strains in LTEE

During this first year of the project, we deleted the *crp* gene in 20 evolved clones (Figure 2, Table 3). This was performed by allelic exchange using a suicide-plasmid methodology routinely used by UJF [Cooper et al., 2008]. The aim was first to construct the *crp* deletion in evolved clones sampled after 40,000 generations from each of the twelve populations (because at the start of this project, it was the last time point for which we had available genome sequences). For three populations, named Ara-1, Ara-5 and Ara+4, we were unable, for unknown reasons, to construct the deleted strains. However, we could generate the *crp* deletions in earlier time points for those

populations. Second, we wanted for some focal populations to have time series experiments to study the dynamics of the changes in regulatory networks, and we also deleted *crp* in clones from earlier time points. The list of available strains is given in Table 3 and represented schematically in Figure 2. In sum, we have now *crp*-deleted strains in all 12 populations, and for three of them at different time points (Figure 2, Table 3).

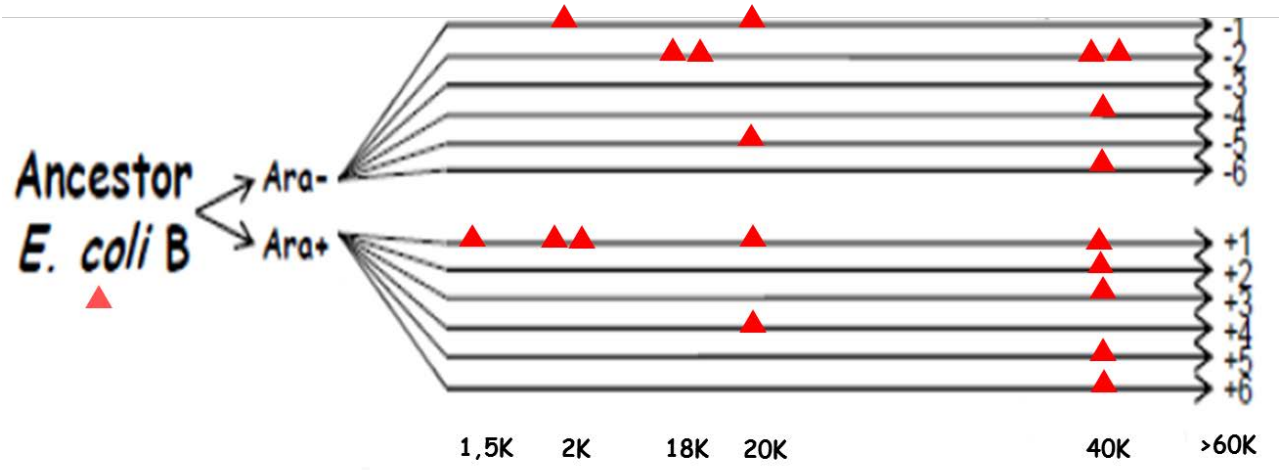

Figure 2: Construction of *crp*-deletions in evolved clones sampled from the LTEE. The deleted strains are represented by red triangles (K: x1000 generations).

3.1.2. Identification of combinations strains-environments affecting bacterial robustness

The effect of the *crp* deletion on growth was investigated in all populations by direct plating on rich medium plates. Deleting *crp* had a drastic effect on growth on solid medium in all evolved clones. Hence, all deleted strains produced small colonies, indicative of growth defects. To quantify this behavior in more details, we started to investigate the effect of the *crp* deletions in two populations: Ara-1 and Ara+1 by cultures in liquid media, including the minimal glucose medium used in the evolution experiment and alternative environments.

Deleting *crp* in evolved clones sampled after 20,000 generations from each Ara-1 and Ara+1 populations severely affected the growth rates in minimal glucose medium (Figure 3). These growth defects were much more severe in the evolved genetic backgrounds than in the ancestor, implying that mutations substituted during evolution in both populations interacted with the *crp* deletion. To investigate when these epistatic interactions occurred during evolution, the *crp* deletion was constructed in evolved clones sampled earlier during evolution of the two populations.

The results shown in Figure 3 revealed that the severe growth defects were already detected after 1500 and 2000 generations in Ara+1 and Ara-1, respectively. Therefore, in both populations, mutations that were substituted early during evolution interacted epistatically with the *crp* deletion.

However, these changes in the epistatic interactions were completely reversed after 40,000 generations of evolution in population Ara+1. Indeed, deleting *crp* in the 40,000-generation clone in Ara+1 resulted in increased growth rates, a process that is currently under investigation (Figure 3). These results revealed a high dynamics of the CRP-controlled regulon, and therefore of the regulatory network, during long-term evolution.

Table 3: Clones derived from the LTEE in which *crp* deletions have been constructed.

Generation	Clone	Population	Growth rate	Status
Ancestor	REL606	*E. coli*	Done, decreased	Done, evolution for suppressor and evolvability analyses In progress, growth in selective and alternative media to measure robustness
1500	1062A	Ara+1		In progress, growth in selective and alternative media to measure robustness
2000	1158A	Ara+1	Done, decreased	Done, evolution for suppressor and evolvability analyses Done, growth in selective and alternative media to measure robustness
	1158C	Ara+1	Done, decreased	Done, evolution for suppressor and evolvability analyses Done, growth in selective and alternative media to measure robustness
	1164A	Ara-1	Done, decreased	Done, evolution for suppressor and evolvability analyses Done, growth in selective and alternative media to measure robustness Done, identification of interfering mutations
18,000	7809	Ara-2S		In progress, growth in selective and alternative media to measure robustness and phenotypic innovation
	7810	Ara-2L		In progress, growth in selective and alternative media to measure robustness and phenotypic innovation
20,000	8603A	Ara+4		In progress, growth in selective and alternative media to measure robustness
	8597A	Ara-5		In progress, growth in selective and alternative media to measure robustness

	9282A	Ara+1		In progress, growth in selective and alternative media to measure robustness
	8593A	Ara-1		Done, growth in selective and alternative media to measure robustness Done, identification of interfering mutations
40,000	11008	Ara+1		In progress, growth in selective and alternative media to measure robustness
	10950	Ara+2		In progress, growth in selective and alternative media to measure robustness
	10953	Ara+3		In progress, growth in selective and alternative media to measure robustness
	10957	Ara+4		Discarded, not possible to construct the *crp* deletion despite many trials; *crp* deletion obtained in a 20,000-generation clone of the same population
	10982	Ara+5		In progress, growth in selective and alternative media to measure robustness
	10985	Ara+6		In progress, growth in selective and alternative media to measure robustness
	10939	Ara-1		Discarded, not possible to construct the *crp* deletion despite many trials; *crp* deletion obtained in a 2,000-generation clone and in a 20,000-generation clone of the same population
	11036	Ara-2S		In progress, growth in selective and alternative media to measure robustness and phenotypic innovation
	11035	Ara-2L		In progress, growth in selective and alternative media to measure robustness and phenotypic innovation

	40K-A	Ara-3		Discarded, not possible to construct the *crp* deletion despite many trials
	10944	Ara-4		In progress, growth in selective and alternative media to measure robustness
	40K-C	Ara-5		Discarded, not possible to construct the *crp* deletion despite many trials; *crp* deletion obtained in a 20,000-generation clone of the same population
	11005	Ara-6		In progress, growth in selective and alternative media to measure robustness

To extend these analyses to other environments, we measured the growth abilities of parental and *crp*-deleted strains using GN2 Biolog plates (AWELinternational, BLAIN, France) that contain 96 different carbon sources (Figure 4). We measured the effect of the *crp* deletion on the growth in these alternative environments in the ancestor and two evolved clones sampled after 2000 and 20,000 generations from population Ara-1 (these analyses are currently being extended to other genetic backgrounds). Deleting *crp* drastically reduced the catabolic breadth of each of the three strains. However, the changes in catabolic breadth were different in the ancestral and evolved genetic backgrounds. Therefore, at least for population Ara-1, the *crp* deletion affected growth more severely in the evolved clones than in the ancestor in the minimal glucose medium in which the evolution occurred, and differentially affected the ecological niche breadth in alternative environments. This suggests that evolution in the glucose environment strongly selected a particular structure of the regulatory network and that disturbing this structure (here by deleting *crp*) resulted in differential robustness of the evolved clones compared to the ancestor.

We have now started evolution experiments to adapt the *crp*-deleted strains to this minimal medium to see whether and how they can recover their growth abilities. This is the subject of the next deliverable.

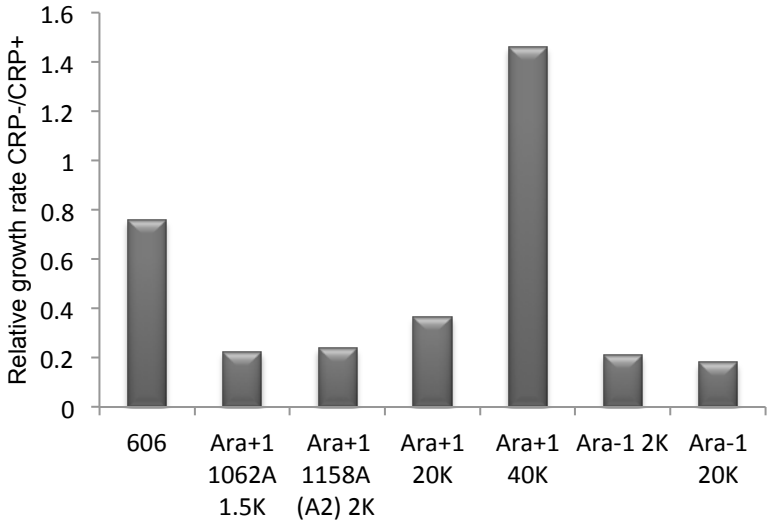

Figure 3: Effect of the *crp* deletion on the growth abilities in evolved clones from populations Ara-1 and Ara+1 (K: x1000 generations). The relative growth rate the each *crp*-deleted strain compared to the corresponding parental *crp*$^+$ strain is given.

A1 eau	A2 α-cyclodextrine	A3 dextrine	A4 glycogène	A5 Tween 40	A6 Tween 80	A7 N-acétyl-D-galactosamine	A8 N-acétyl-D-glucosamine	A9 adonitol	A10 L-arabinose	A11 D-arabitol	A12 cellobiose
B1 i-érythritol	B2 D-fructose	B3 L-fucose	B4 D-galactose	B5 gentiobiose	B6 α-D-glucose	B7 m-inositol	B8 α-lactose	B9 α-D-lactose lactulose	B10 maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β-méthyl D-glucoside	C3 psicose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-tréhalose	C9 turanose	C10 xylitol	C11 méthyl pyruvate	C12 mono-méthyl succinate
D1 acide acétique	D2 acide cis-aconitique	D3 acide citrique	D4 acide formique	D5 acide D-galactonique lactone	D6 acide D-galacturonique	D7 acide D-gluconique	D8 acide D-glucosaminique	D9 acide D-glucuronique	D10 acide α-hydroxy butyrique	D11 acide β-hydroxy butyrique	D12 acide γ-hydroxy butyrique
E1 acide p-hydroxy phényl-acétique	E2 acide itaconique	E3 acide α-kéto butyrique	E4 acide α-kéto glutarique	E5 acide α-kéto valérique	E6 acide D,L-lactique	E7 acide malonique	E8 acide propionique	E9 acide quinique	E10 acide D-saccharique	E11 acide sebacique	E12 acide succinique
F1 acide bromo succinique	F2 acide succinamique	F3 glucuronamide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-alanyl-glycine	F8 L-asparagine	F9 acide L-aspartique	F10 acide L-glutamique	F11 acide glycyl-L-aspartique	F12 acide glycyl-L-glutamique
G1 L-histidine	G2 hydroxy L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phényl alanine	G6 L-proline	G7 acide L-pyro glutamique	G8 D-serine	G9 L-serine	G10 L-thréonine	G11 D,L-carnitine	G12 acide γ-amino butyrique
H1 acide urocanique	H2 inosine	H3 uridine	H4 thymidine	H5 phényl éthylamine	H6 putrescine	H7 2-amino éthanol	H8 2,3-butanédiol	H9 glycérol	H10 D,L-α-glycérol phosphate	H11 glucose-1-phosphate	H12 glucose-6-phosphate

Figure 4: Representation of a GN2 Biolog plate with the 95 carbon sources.

3.2. Articles published within the framework of this study

Raeside C, Gaffé J, Deatherage DE, Tenaillon O, Briska AM, Ptashkin RN, Cruveiller S, Médigue C, Lenski RE, Barrick JE, Schneider D. 2014. Large chromosomal rearrangements during a long-term evolution experiment with *Escherichia coli*. *mBio* 5(5):e01377-14.

4. Conclusion

A large collection of TEV and *E. coli* strains has been produced during the first year of the project. In TEV, these strains have been designed to produce variation and shuffling of their genome structure (including gene duplication, essential gene deletion, insertion of foreign genes, including useless and functional ones). In *E. coli*, the engineered strains have been deleted for *crp*, a gene encoding a global regulator known to regulate the transcription of more than 150 genes. Perturbation of the regulatory network is therefore the result of such deletions.

For different (and sometimes unknown) reasons, some constructed strains were unstable or impossible to produce. Unstability or non-viability in *N. tabacum* was indeed found for 8 of the engineered TEV strains. In the *E. coli* model, we were unable to construct the *crp* deletion in 3 of the 12 *E. coli* populations at generation 40,000 despite intensive and repeated trials (populations Ara-1, Ara-3 and Ara-5). To overcome this limitation and owing to the availability of fossil records over evolutionary time, the *crp*-deletions were constructed at generation 20,000 in the two populations Ara-1 and Ara-5, whereas it was still impossible to obtain the engineered strain in population Ara-3 at that time point. Moreover, to gain insights into the dynamics of the early changes in regulatory networks, and in addition to the initial workplan, we constructed *crp*-deletions in strains isolated at 1500, 2000 and 20,000 generations from population Ara+1 (in addition to the deletion constructed in the 40,000-generation clone).

Despite these limited difficulties in the strain construction process, WP1 is not at risk for subsequent experiments and analyses. Indeed, enough engineered strains have been produced in both models and, in some cases, missing strains have been replaced by *crp*-deleted constructs at other time points in *E. coli* (additional constructed strains have even been produced), and by close constructions in TEV.

Experimental evolution lineages that use some of the produced strains as ancestors have already been initiated and even finished for a few. In the *E. coli* model, chromosomal rearrangements have been mapped and their effects on fitness tested, which already corresponds to a later deliverable. The results obtained so far led to two publications in renowned international journals [Tromas et al., 2014; Raeside et al., 2014] and a third publication is in revision.

All the strains mentioned in this document have been produced by partner 5 (CSIC, Valencia, Spain) and partner 2 (UJF, Grenoble, France). They are conserved and managed by the groups that engineered them: all TEV strains are conserved and studied by CSIC; all *E. coli* strains are conserved and studied by UJF. They are freely available for scientific purpose upon request to the relevant partner.

5. References

Cooper, T. F., S. K. Remold, R. E. Lenski, and D. Schneider. 2008. Expression profiles reveal parallel evolution of epistatic interactions involving the CRP regulon in *Escherichia coli*. *PLoS Genetics* 4(2):e35.

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