EvoEvo Deliverable 1.3
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**EvoEvo Deliverable 1.3**

**Analysis of evolvability (part 1)**

Due date: M22 (September 2015)

Person in charge: S. Elena

Partner in charge: CSIC

Workpackage: WP1 (Experimental observation of EvoEvo in action)

Deliverable description: Analysis of evolvability (part 1): evolvability: correlation between viral evolvability and robustness; correlation between rates of virus evolution and genome architecture; correlation between bacterial evolvability and restructuration of regulatory networks.

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1. **Introduction**

A system is said to be evolvable if it can be modified through genetic change in a way that enhances survival and reproduction. For natural selection to act, the system must show heritable phenotypic variation. Yet genetic robustness implies that the system produces little phenotypic variation in response to genetic variation. Therefore, robustness might limit evolutionary optimization and innovation (Lenski *et al.* 2006). In this vein, theoretical work has postulated that buffering mechanisms can lead to maladaptation compared to what would be achieved in their absence (Frank 2007). Also, the analysis of gene expression noise in yeast suggests that noise control may indirectly increase mutational robustness, which might in turn hamper evolvability at the level of gene expression (Lehner 2008). On the other hand, genetic robustness facilitates the accumulation of neutral or nearly neutral variation by relaxing the intensity of natural selection. This accumulated diversity can become visible to selection upon changes in the environment or genetic background, and thus be the source of evolutionary innovation. Computer simulations on simple population genetic models predict that genetic robustness can sometimes facilitate access to new adaptive peaks provided that occasional failures of robustness may occur (Kim 2007). The view that robustness can foster evolvability has also been supported by lattice protein models and PCR-based mutagenesis experiments showing that protein variants with increased thermostability have increased genetic robustness and are more likely to evolve new catalytic capabilities (Bloom *et al.* 2006).

This Task builds on the preliminary results of Task 1.1 to study evolvability and its interactions with robustness. It is divided in two sections that will address evolvability at two different levels using the most appropriate experimental model: evolvability at the population level in the TEV model, and at the regulatory network level in the *E. coli* model.

2. **Task 1.2 Evolvability at the population and regulatory network levels**

2.1. **Section 1: Evolvability at the population level in the TEV experimental model**

The evolvability of the different TEV genotypes constructed for Task 1.1 (Deliverable 1.1) was evaluated. To do so, we have quantified the relative rate of fitness change during experimental evolution on the natural host *N. tabacum* and on alternative hosts (*Nicotiana benthamiana*, *Datura stramonium*, *Helianthus annuus*, and *Spinacea oleracea*). Here we will describe only the results obtained for the natural host, tobacco. Results for the alternative hosts will be described in part 2, expected for future deliverables.

Ten lineages of each of the eight viral genotype organizations engineered were maintained by undiluted serial passages in tobacco. Infectious dosages were equivalent in all transmission events and the impact of genetic drift was minimized to make mutation and selection become the dominant evolutionary forces operating in these experiments. This was achieved by using highly concentrated virus preparations and maximizing the number of infection foci per plant by mechanical inoculation thus minimizing transmission bottlenecks (Zwart *et al.* 2011). Three 9-weeks passages were performed in all cases. These demographic conditions have been shown to optimize the effect of selection in fixing beneficial alleles and improve the fitness of reengineered genomes (Zwart *et al.* 2014). The fitness of the ancestral and resulting evolved lineages was
evaluated by means of competition experiments against a common competitor (TEV-mCherry) as follows. All ancestral and evolved viral lineages were again normalized to the sample with the lowest concentration, and 1:1 mixtures of engineered genome equivalents was made with TEV-mCherry. The mixtures were mechanically inoculated on *N. tabacum* plants, using three independent plant replicates per viral lineage. The plant leaves were collected at 7 dpi, and stored at -80 °C. RNA was extracted from 100 mg homogenized tissue as described above. RT-qPCR for the *CP* gene was used to determine total viral accumulation, and independent RT-qPCR reactions were also performed for the *mCherry* sequence using specific primers. The ratio of the evolved and ancestral lineages to TEV-mCherry (*R*) is then \( R = \frac{n_{CP} - n_{mCherry}}{n_{mCherry}} \), where \( n_{CP} \) and \( n_{mCherry} \) are the RT-qPCR measured copy numbers of *CP* and *mCherry*, respectively. Then we can estimate the within-host competitive fitness as \( W = \sqrt[3]{R_t/R_0} \), where \( R_0 \) is the ratio at the start of the experiment and \( R_t \) the ratio after \( t \) days of competition.

Figure 1 shows the results from these experiments. Each bar represents the grand mean of the 10 independent evolution lineages. Figure 1A shows the fitness values of ancestral and evolved viruses; Figure 1B shows the relative change in fitness, a measure of evolvability. Differences exist among genetic backgrounds in their evolvability (1-way ANOVA, \( P < 0.01 \)), with TEV-eGFP being the most evolvable and TEV-Nlb2, TEV-Nlb1 and TEV-2b not showing significant changes in fitness relative to the ancestral. Interestingly, TEV-eGFP and TEV-2NlaPro are more evolvable than the wildtype TEV, whereas the genomes of TEV-2Nlb2 and TEV-2Nlb1 are as evolvable as the wildtype genome.

We can conclude from these experiments that alternative genomic organizations differ in their evolvability, some being more and some less evolvable than the wildtype TEV genome.
Finally, we sought for the association between the robustness of these genotypes (evaluated in Task 1.1 Section 3) and their evolvability (evaluated here). It has been suggested that evolvability and robustness shall be negatively correlated in the short evolutionary time (because robustness mechanism will buffer the effect of beneficial mutations) but positively correlated in the long run.

Figure 1 – Evolvability of TEV strains with different genomic architectures. (A) Differences in fitness of ancestral and evolved genomes. (B) Relative change in fitness (evolvability) for each genome. Error bars represent the standard deviation computed from 10 independent evolution lineages.
(because robustness allows accumulation of variation and exploration of distant regions of genotypic spaces) (Elena & Sanjuán, 2008). Figure 2 illustrates the relationship between these values. No significant association between these two traits has been observed for the four genotypes so far fully analyzed (Spearman $r_S$ test, $P > 0.05$). Interestingly, the wildtype TEV genome is the most robust one but occupies an intermediate location in the ranking of evolvability. There are two possible explanations for this lack of association. Firstly, it is a simple statistical problem, owed to the very small sample size. This may fix as we add more data to the analysis. Secondly, may be observing the composed effect of short- and long-term effects, flating off any significant trend.

![Figure 2](image-url) - Lack of correlation between evolvability and robustness for the four engineered TEV genomes analyzed.

2.2. **Section 2: Evolvability at the regulatory network level in the *E. coli* experimental model**

This deliverable included evolution experiments by evolving crp-deleted strains in environments, determined during task 1.1, in which they have large fitness defects (D1.3 due at M20). The objective will be to select evolved clones with restored fitness and to sequence their genome to investigate the underlying molecular mechanisms (D1.5, due at M36). These experiments will allow us to understand how regulatory networks can be rewired after perturbation to provide new adaptive abilities to bacterial cells.
2.2.1. Evolution experiments to recover fitness (D1.3 and D1.4, due at M20 and M22, and finished at M18)

We initiated evolution experiments using crp-deleted strains as ancestors. They were propagated in a constant glucose-limited environment (DM1000) where we previously showed (task 1.1) that they had strong growth defects. The aim of these evolution experiments is to address whether and how they will recover their growth abilities. Four crp-deleted strains were propagated by daily transfers for 100 generations in DM1000 (Figure 3). The results that we gathered in Deliverable D1.2 targeted the choice of the four strains in which crp was deleted: the ancestor clone, one 2000-generation evolved clone from each of the Ara-1 and Ara+1 populations, and the ancestral clone in which we moved the Ara-1 topA allele that interferes with the crp deletion (see Deliverable 1.2 section 2.2.3). Five replicate populations were propagated for each of the four strains. Mixed populations were sampled after 33, 66 and 100 generations and frozen at -80°C. Moreover, 3 individual clones were isolated from the 100-generation mixed population and also frozen at -80°C.

We then measured the growth rate of each of the evolved clones isolated from each population after 100 generations and compared it to the one of their direct ancestor (Figure 4). Most evolved clones were able to increase or restore their growth rates.

![Figure 3 - Evolution experiments using four crp-deleted strains as ancestors (K: x1000 generations).](image-url)
2.2.2. Identification of compensatory mutations (D1.5, due at M36 and started during the last six months)

We selected the clones: ancestor topA\(^{-1}\)Δcrp1A, ancestor topA\(^{-1}\)Δcrp4A, ancestor topA\(^{-1}\)Δcrp5C, Ara+1 2KΔcrp1A, Ara+1 2KΔcrp2B and Ara+1 2KΔcrp5A (Figure 4) for further genomic analyses. We extracted genomic DNA from each of these 6 clones. Their genome sequences were determined and analyzed using the Bresq pipeline (Deatherage & Barrick, 2014). Based on these genome sequences, we identified the mutations that were substituted during evolution and that may be involved in the restoration of the growth rate defects associated with the crp deletion in each of the four genetic backgrounds (Table 1). A high level of parallelism was observed with mutations identified in identical genes in several independently evolved clones from different ancestral strains. A high level of parallelism has been frequently observed associated with positive selection (Crozat et al. 2005, Pelosi et al. 2006, Barrick et al. 2009), thereby suggesting that the mutations we identified may confer higher fitness. In particular, mutations in the ptsG gene, involved in glucose transport, were selected for in 5 of the 6 analyzed evolved clones. Candidate mutations in ptsG from the five evolved clones ancestor topA\(^{-1}\)Δcrp1A, ancestor topA\(^{-1}\)Δcrp5C, Ara+1 2KΔcrp1A, Ara+1 2KΔcrp2B and Ara+1 2KΔcrp5A are currently being moved in the relevant ancestral chromosome together with the crp deletion to demonstrate that they are involved in growth restoration. These results should allow us to understand how evolvable regulatory networks are and to determine the molecular mechanisms underlying changes in epistatic interactions within
regulatory networks. Indeed, the mutations in \( \text{ptsG} \) occurred in its promoter region in sites known to be bound by transcription factors. To investigate the changes in molecular mechanisms associated with these mutations, we are now planning to analyse their effects on the transcription level of \( \text{ptsG} \), the glucose transport rate inside the cells, and the binding of transcription factors in that regulatory region. Therefore, we will have a complete understanding, at the evolutionary, physiological, molecular and biochemical levels, of the rewiring of this regulatory network. This deliverable, which is due at M36 is therefore well advanced and no particular problem is foreseen here.

### Table 1 – Mutations identified by genome sequencing in evolved clones derived from \( \text{crp} \)-deleted strains

<table>
<thead>
<tr>
<th>Ancestral strain</th>
<th>Evolved clone</th>
<th>Gene</th>
<th>Type</th>
<th>Change*</th>
<th>Gene function</th>
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<tbody>
<tr>
<td>Ara+1 2K ( \Delta \text{crp} )</td>
<td>Ara+1 2K ( \Delta \text{crp1A} )</td>
<td>( \text{ptsG} )</td>
<td>Deletion</td>
<td>15 bp</td>
<td>glucose PTS permease</td>
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<tr>
<td></td>
<td>Ara+1 2K ( \Delta \text{crp2B} )</td>
<td>( \text{ptsG} )</td>
<td>Deletion</td>
<td>15 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ara+1 2K ( \Delta \text{crp5A} )</td>
<td>( \text{ptsG} )</td>
<td>Deletion</td>
<td>15 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>( \text{yihS} )</td>
<td>IS_insertion</td>
<td>IS150</td>
<td>sulfoquinovose isomerase</td>
</tr>
<tr>
<td>Ancestor ( \text{topA}^{-\Delta \text{crp}} )</td>
<td>Ancestor ( \text{topA}^{-\Delta \text{crp1A}} )</td>
<td>( \text{ptsG} )</td>
<td>Mutation</td>
<td>C( \rightarrow )T</td>
<td>glucose PTS permease</td>
</tr>
<tr>
<td></td>
<td>Ancestor ( \text{topA}^{-\Delta \text{crp4A}} )</td>
<td>( \text{manX} )</td>
<td>Mutation</td>
<td>C( \rightarrow )T</td>
<td>mannose PTS permease</td>
</tr>
<tr>
<td></td>
<td>Ancestor ( \text{topA}^{-\Delta \text{crp5C}} )</td>
<td>( \text{ptsG} )</td>
<td>Mutation</td>
<td>C( \rightarrow )T</td>
<td>glucose PTS permease</td>
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*All mutations are in the transcriptional regulatory regions of each gene.

### 3. Conclusion

Evolvability has been studied at two different levels using the most appropriate biological model. At the genomic level, experiments with the TEV model have shown that:

- Different genomic organizations affect the evolvability of TEV, with some organizations being more evolvable than the wildtype and other less so.
- No significant association has been observed between robustness and evolvability, although this may be due to the reduced sample size of these experiments.
At the regulatory level, experiments with *E. coli* have shown that:

- Perturbing the *crp* regulatory network had a drastic growth defects in a glucose-limited environment. We showed that this defect can be restored after only 100 generations of propagation in that environment. Therefore, regulatory networks are highly evolvable.
- We identified the mutations allowing growth restoration and evolvability of the network. They affect *ptsG*, a gene involved in glucose transport across bacterial membranes. These mutations are currently investigated to investigate their molecular effects.

4. References


