The preprint by Grohens, Meyer, and Beslon raises the questions i) of the potential role of the coupling between DNA supercoiling and transcription in the evolution of genome organization, and ii) of the possible role of this coupling in the evolutionary selected transcriptional regulatory mechanisms that aim to modulate the expression of a large number of genes, either through repression or activation, in response to environmental changes.

The subject is of great importance since there is still no tangible evidence that DNA supercoiling, particularly the transcription-supercoiling coupling, is used by cells to adapt gene expression to environmental changes. Indeed, the results up to today show systematic correlations, but correlation does not imply causality, especially given that DNA supercoiling is inherent to the act of transcription.

Personally, I find the overall message of the article interesting, namely, that only the coupling between transcription and supercoiling enables a genome to evolve in such a way that the evolved genome responds differentially to two antagonistic environments. Furthermore, the resulting organization of evolved genomes appears to be non-trivial, which could explain the systematic presence of certain organizational motifs of genomes as observed by several groups (see point B, thouh).

A. However, I believe several points need to be improved, deserving to be analyzed more deeply or at least discussed. I have identified four major points, three of a more technical nature and one concerning the introduction of the paper.

1. The first technical point concerns the complexity of the model. The authors mention in this regard that they "kept [their] model as simple as possible in order to obtain easily interpretable results." First, I believe it is important to emphasize that the rules of influence of a gene on its neighbor are not yet established, and as such, simplicity is necessary to identify phenomena that should not depend on the details of the model. Furthermore, in this context, it is difficult to understand the choice of such a complex description as the one involving four equations for the link between supercoiling and transcription, which implies five parameters (c, dmax, epsilon, m, sigma_0). These parameters should impact the results discussed in the paper, but no impact study is presented. For example, a simple step function for the expression dependence on sigma (which equals 1 if sigma > sigma_0 and 0 otherwise) would allow the elimination of the two parameters epsilon and m.
2. The second technical point concerns the value of dmax that the authors take as equal to 5 kb. This value is justified by the fact that, in this case, the impact of sigma is felt up to 10 kb, which corresponds to the length of the topological domains highlighted by the Cozzarelli group in the 2000s [Postow et al., 2004]. The problem here is that the measurement by Postow et al. is more related to an effective emerging distance, indirectly mentioned by the authors when they raise the question of what the size of the neighborhood is that needs to be considered to explain the expression of a gene (section 2.3) or the properties of the generated regulatory graph, rather than an interaction distance without taking the genetic context into account. An important question that would have deserved to be discussed is what value of dmax would allow, for example, to obtain minimal network sizes (Figure 7) on the order of 10 genes (i.e., 10 kb). Actually, the choice of long-range interaction remains to be physically justified, considering that it has been shown that transcribing RNAPs act as topological barriers (work by Higgins in particular, not mentioned in the article).
3. The third technical point concerns the absence of discussion of results when $\delta \sigma=0$ in the problem of creating genomes that are able to differentially express between two environments. In fact, on two occasions, the authors mention that their results remain the same when $\delta \sigma$ is 10 or 100 times smaller, which could be interpreted as a very general property of the framework used. My personal analysis is that at least in one case, that of the system's ability to evolve towards a system capable of expressing itself differentially depending on two environments (line 226), has little to do with the value of $\delta \sigma$. Indeed, the fact that genes are expressed differentially in two environments seems to be a direct consequence of the presence of organizational motifs, as discussed by the authors (see comment B below, though), rather than the values of $\delta \sigma$. The introduction of $ \delta \sigma$ then allows the symmetry between A and B to be broken and leads to an interesting discussion of the effect of activation by relaxation, which ultimately is relaxation by the genetic context, as discussed in the article.

4. The introduction contains several errors and inaccuracies that should be corrected:
   - Line 27: "Both writhing and twisting are referred to as DNA supercoiling" is not correct. One can have both a non-zero writhe and a non-zero twist but still have a relaxed linking number, i.e., no supercoiling. DNA supercoiling should be properly introduced by mentioning the linking number.
   - Line 42: "and rotating the DNA strands" is not correct. Bacterial topoisomerases are about strand passages — see e.g. McKie, S.J., Neuman, K.C., Maxwell, A., 2021. DNA topoisomerases: Advances in understanding of cellular roles and multi-protein complexes via structure-function analysis. BioEssays 43, 2000286.
   - Line 50: the current hypothesis (formulated by Liu and Wang in 1987) is that cytoplasmic friction acts on the complex made by the polymerase AND the translating ribosomes. In their paper, Liu and Wang actually estimate that the friction acting on the RNAP alone would be too small to prevent it from rotating.
   - Line 53: so far, there is no evidence that a DNA-bound protein can create a barrier. There is strong in vitro evidence that loops create such barriers. There is also evidence that transcribing RNAPs create such barriers (work by Higgins should be cited here).
   - Line 55: "The level of DNA supercoiling can additionally be affected by numerous environmental factors": this sentence suggests that there are physical mechanisms other than the action of topoisomerases, the action of the RNA polymerases, and the topological barriers due to structural constraints of the chromosome that can directly modify the overall supercoiling level. However, what is discussed is the observed change of supercoiling in different conditions, which can be explained as a consequence of these very factors. It would therefore be worth better distinguishing the known physical mechanisms that explain these variations, on the one hand, and the biological processes that are associated with these changes. This problem is actually directly related to the problem of distinguishing causality from correlation.
   - The introduction and conclusion suggest that the coupling between transcription and supercoiling dates from (Meyer and Beslon, 2014). This is misleading as this notion was first introduced in the late 1980s (see e.g. Pruss & Drilca, DNA supercoiling and prokaryotic transcription, Cell 1989) and was greatly discussed and investigated in the following years (see e.g. Lilley, Chen & Bowater, DNA supercoiling and transcription: topological coupling of promoters, Quarterly Reviews of Biophysics, 1996). (Meyer and Beslon, 2014) provided, for the first time, a mathematical model of such coupling with some interesting quantitative predictions, which they discussed more particularly in the context of Drosophila melanogaster. Along this line, it is important to clarify in lines 106 to 110 that these are predictions of a mathematical model. Reality is more complex (see e.g. Kim et al., Cell, 2019).
   - Line 130: (Peter et al., 2004) did not report any clustering effect associated with their supercoiling-sensitive genes. Quite the contrary, they actually conclude: "The supercoiling-sensitive genes are functionally diverse and widely dispersed throughout the chromosome."

B. Next, it seems to me that a relatively central explanation, analysis, and discussion are missing from the paper. Indeed, it appears clear that the phenomenology of genes A results from the presence of patterns of the type AAB with divergent AA and convergent A and B (visible in Figure 1). My issue is that the authors discuss divergent AA and convergent AB but not AAB, even though it is this property, it seems to me, that explains how the evolved genome is capable of responding to two antagonistic environments. One could then wonder what would happen if instead of two environments, there were three or more environments to satisfy. One could also inquire whether these patterns are present in genomes and could account for some of the observations mentioned by the authors.

C. Other comments:

- Line 17: The term "fine control" is not justified to me (see also "fine-tune" in line 465).
- Line 20: σ_basal can take a wide range of values, depending on conditions. I would indicate that -0.06 is a typical value.
- Line 25: Supercoiled DNA forms super-structures that are more complex than simple loops.
- Line 77: The sentence suggests that the change of supercoiling controls the expression of pathogenic genes in the context of D. Dadantii. I do not think it has been demonstrated, like in any other system.

- Paragraph from line 111: These results should be contrasted with other results such as those by (Kim et al., Cell, 2019).
- Line 138: The sentence suggests that the study by (Junier and Rivoire, 2016) focused on E. coli versus B. subtilis. However, the analysis was more global.
- Paragraph from line 79: I think it would be appropriate to mention a work by Luis Serrano's group, which actually quantifies the contribution of supercoiling in the reduced genome *M. pneumoniae*: Yus et al., Determination of the Gene Regulatory Network of a Genome-Reduced Bacterium Highlights Alternative Regulation Independent of Transcription Factors, Cell Systems, 2019.
- Line 177, about the system of equations: Is it obvious there exists a unique solution?
- Line 178: It would be appropriate to provide a more didactic presentation of the system. In particular, it would be worth mentioning the hypotheses considered. For instance: continuous description (neglect of stochastic effects), linear dependence of the expression of one gene with respect to the distance of where supercoiling varies, neglect of RNAPs acting as topological barriers, etc.
- Line 182-183: To what extent would the genomic inversion rates impact the results discussed here?
- Line 184: Paragraph "Evolution of Environment-Specific Gene Expression Levels": It would be worth mentioning upfront that there are 20 genes in each class.
- Figure 1 & 3: Titles (Env A, Env B) would facilitate the reading.
- Figure 4: Non-informative title.
- Legend of Figure 4: "Random genome" would be more informative.
- Page 11: The first paragraph starting at line 227 is difficult to understand, due to the (apparently unnecessary) complexity of the model.
- Figure 4: It is not totally clear how these curves have been produced. This could be explained in the Methods.
- The observation that "Pairwise Interactions Do not Recapitulate the Regulatory Network" is actually rather expected since the model is not built from nearest neighbor interactions, i.e., dmax is large with respect to the size of a gene (see also my comment A.2.). Any conclusion would require a comparative analysis with a Random genome.
- Lines 404-405: Is it really that surprising? Since the network is, by construction, of a circular type (Figure 9) and since any strong local effect would connect the ring.
- Line 515: "reaction norm of gene promoters"?
- Line 539: The authors suggest that their framework can be considered a reasonable model (the first one) of the interplay between supercoiling and transcription in bacterial genomes. I would be more modest on this aspect as there are numerous simplifications used in the framework, which raise the question of the relevance of such a model for a realistic situation. I do agree, nevertheless, that this work provides, for the first time to my knowledge, a picture of the importance of the role of DNA supercoiling, and more specifically the coupling between transcription and supercoiling, in the evolution of genome organization and gene regulation.