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For the sake of the Bioeconomy: define what a Synthetic Biology Chassis is!

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ABSTRACT

At the onset of the 4th Industrial Revolution, the role of synthetic biology (SynBio) as a fuel for the bioeconomy requires clarification of the terms typically adopted by this growing scientific-technical field. The concept of the chassis as a defined, reusable biological frame where non-native components can be plugged in and out to create new functionalities lies at the boundary between frontline bioengineering and more traditional recombinant DNA technology. As synthetic biology leaves academic laboratories and starts penetrating industrial and environmental realms regulatory agencies demand clear definitions and descriptions of SynBio constituents, processes and products. In this article, the state of the ongoing discussion on what is a chassis is reviewed, a non-equivocal nomenclature for the jargon used is proposed and objective criteria are recommended for distinguishing SynBio agents from traditional GMOs. The use of genomic barcodes as unique identifiers is strongly advocated. Finally the soil bacterium *Pseudomonas putida* is shown as an example of the roadmap that one environmental isolate may go through to become a *bona fide* SynBio chassis,

Introduction

The language of physics and engineering uses clear definitions of forces, measures, and units to be precise and unequivocal. If one says ampere, kilometer, gravity or screw pitch it is known exactly what the term means. In contrast, the jargon of synthetic biology (SynBio), which aims at transforming biology to an engineering discipline is peppered with words and metaphors which seem to be more a declaration of intentions (or wishes) than an accurate description of the items named thereby. One of the central notions of contemporary SynBio is that of *chassis* [1–4] a term that evokes the basic frame of a car to which a number of components can be added in response to specifications and/or customers' desires (from French: *châssis* = frame; latin *capsa* = container, box). By the same token, the word started to be used in the early 2000s by the incipient SynBio community of the time as a somewhat humorous and engineering-sounding description of the biological host used as the recipient of recombinant DNA — which by that time meant nearly exclusively *Escherichia coli*. The word chassis (and the powerful metaphor embodied in it) became an instant success and was quickly incorporated into the habitual discourse of SynBio-as-engineering. However, the word has acquired new meanings over the years and has been used in many different contexts beyond its original and somewhat modest significance.

The prevailing meaning of chassis is that of a more or less improved host for genetic constructs whether in bacteria yeast, fungi, archaea, animal or plant cells. But the term has also been applied to organisms with edited genomes for enhancing this or that trait of interest as well as bacteria with minimized DNA contents, including altogether synthetic genomes [5,6]. Moreover, cell-free systems [7], reconstructed vesicles and nucleoid-less cells, i.e. with no DNA [8], have also been described with the same term. In a further screw turn, subsets of biochemical reactions either implemented in vitro or simulated in silico are also often referred to as chassis. The meaning of the word has thus quickly undergone a process of polysemic diversification to the point that the metaphor is kept in all cases but the precise meaning has become increasingly blurred. This has not been an issue thus far, but the new scenarios that SynBio starts to penetrate demand clarification and even a definition of the term that end-users can understand without any

Abbreviations: SynBio, synthetic biology; GMO, genetically modified microorganism; SBA, synthetic biology agent; rRNA, recombinant DNA; GRAS, generally regarded as safe; ERA, environmental risk assessment; QPS, qualified presumption of safety; EFSA, European Food Safety Authority; FDA, US Food and Drug Administration; HGT, horizontal gene transfer.

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ambiguity. There are good reasons for this.

The first motivation to revisit the concept is the avalanche of proposed species and strains that claim recognition as SynBio platforms [9–17]. Recent publications list dozens of bacterial types — from plain isolates to highly edited genomes — that are presented as such, with dramatic differences in the degree of optimization for bioengineering purposes. There is thus a legitimate need to differentiate isolates just bearing interesting properties from those strains that qualify as *bona fide* SynBio chassis. The second reason is the fuzzy line between organisms that are just good at receiving and maintaining recombinant DNA (i.e. host-vector systems) and those specifically named as SynBio chassis. This apparently futile frontier between the two has important regulatory consequences — as any other criterion to differentiate genetically modified organisms (GMOs) from what we propose to design as SynBio agents (SBAs). While the term *agent* may contemporarily evoke military connotations, we argue that the sematic origin of the word describes accurately the purpose and control that is implicit in such a designation. Etymologically, *agent* comes from latin *agens*, the present active participle of *agere*, meaning *drive*, *lead*, *conduct*, *manage*, *perform*, *do*.

Finally, as discussed later, a proper definition of chassis can ease regulatory roadmaps to industrial and regulatory acceptance [18,19]. The need to come up with good definition of a SynBio chassis in fact stems from demands by regulatory bodies. As deeply engineered agents leave the laboratory for potential use in industry and the environment, they start falling under the radar of agencies that that provide risk assessment advice on products used for the agri/food/feed chain e.g. the European Food Safety Authority (EFSA), and even have regulatory authority e.g. the US Food and Drug Administration (FDA).

Clarifying the nomenclature: towards a definition of SynBio chassis

The discussion about chassis would certainly benefit from having objective criteria for distinguishing specialized carriers of synthetic constructs from mere recipients of cloned DNA. In fact, the traditional notion of recombinant DNA host of the late 1970s–early1980s and the more recent SynBio chassis is largely related to the level of knowledge and availability of wet and computational tools for genetic programming, rather than a categorical distinction. As a first approach, one can describe a synthetic biology chassis as *an engineerable and reusable biological platform with a genome encoding a number of basic functions for stable self-maintenance, growth and optimal operation but with the tasks and signal processing components growingly edited for strengthening performance under pre-specified environmental conditions.* This definition needs some qualifications in the context of this article. First, while the term can be applied to many types of biological platforms, including cell-free systems, purified components and even in silico models, we will deal here only with live microorganisms. Second, note that the key of the definition is optimal *performance*, not *minimized genome size* (although deletion of unnecessary functions will certainly cause a degree of genome reduction [20]).

The quest for the optimal chassis has been addressed from various perspectives. In one case, the idea is to start with a well-characterized bacterium (e.g. *E. coli*) and then delete the parts of the genome that are not necessary for growth in a given environmental context. The extant genomes of microorganisms are populated with a large number of DNA sequences that, on first sight, are dispensable and even deleterious for the final application envisioned for the bacterium [20]. For the time being, some of these minimized *E. coli* strains are the best available chassis for the implantation of new genetic circuits. However, beyond a threshold of genomic reduction, smaller chromosomal size is not only accompanied by a growing dependence on the external milieu for survival, but also by the loss of antigens that may render the synthetic agent invisible to an immune system — thereby creating a safety issue. Finally, note that the definition above implies optimal chassis per specific target environment and tasks therein. The concept thus entails that there may not be a best possible version of these microorganisms, but instead one would find a growing series of related upgraded variants, an issue that intersects with the question of the barcoding and digital twinning of SynBio Agents as discussed below.

Also implicit in this definition is the idea that such chassis stem from well understood and characterized natural organisms which have been genetically streamlined [i] to build, maintain and amplify components necessary for deployment of SynBio systems and applications but also [ii] to ease genetic and metabolic interventions and reduce their adverse effects. To this end, such a chassis should be endowed with natural and knocked-in features suited for facilitating optimal performance in specific settings. For this to happen, they should be amenable to an ample and practical engineering toolbox that allows construction and deployment of genetic devices/circuits with a minimum of engineering steps and thus avoids surprise interactions with host functions. Obviously, these criteria overlap with properties already present in many types of bacteria that can host recombinant DNA and be genetically programmed for a variety of fundamental or biotechnological purposes. However, we argue that a SynBio chassis is more than that: to go beyond being a simple recipient of rDNA and move towards the status of *bona fide* SynBio chassis, engineered microorganisms should have progressed through a well-defined roadmap in which each milestone has unequivocally defined properties. Such a "chassiness" roadmap will help scientists to demarcate more rigorously what a SynBio chassis but, more consequentially, it will also help regulators and policymakers. This is because a (limited) number of standardised microbial platforms — along a well-defined and measurable *chassiness* itinerary — will enable a more transparent and robust examination of regulatory fulfilment while simultaneously lifting regulatory burden via streamlined decision-making when it comes to industrial applications or environmental release.

The roadmap from being a rDNA host to a fully-fledged certified SynBio chassis

Fig. 1 summarizes the itinerary proposed in terms of information and modifications needed for upgrading a promising environmental isolate to a fully-fledged standardized SynBio chassis. This roadmap recapitulates and expands earlier proposals in the same direction by [9,10,12,14–16,21,22] and others. Any (preferably non-pathogenic) environmental isolate able to capture exogenous DNA, through transformation or conjugation, and stably maintain it and for which a minimum of genetic tools is available, can be tagged in principle as a recombinant DNA (rDNA) host. The historical example of this category is *E. coli,* but now there are hundreds of species amenable to a suite of genetic manipulations, including pathogens that are manipulated under controlled laboratory conditions. But to become a true chassis, the biological host should be agreeable to and optimized for accommodating complex genetic devices and deploying their encoded properties under specified operational conditions. For this, additional requirements are needed: the complete genetic complement should be known and advanced genetic tools for deep editing be at hand. This should result in a profound knowledge of the energy metabolism (typically through reliable metabolic models), stress resistance and sensitivity to antibiotics and phages. Knowing the ratio of synthetic/engineered DNA vs. natural genetic complement is straightforward in these cases. Furthermore, genetic and evolutionary stability of the resulting constructs is a most desirable trait. This could be enhanced by engineering circuits that somehow punish mutations in the genetic implants or by making cells deficient in endogenous recombination systems. This, in turn, requires specific genome editing methods that do not rely on recombination, such as targetrons [23] or base editors [24]. Up to that point, one can consider a large number of species and strains that can qualify as, or become, SynBio chassis (see e.g. Table 1). Things get more restrictive, however, when strains are destined for actual, large-scale biotechnological applications, as they must meet additional specifications that are not that

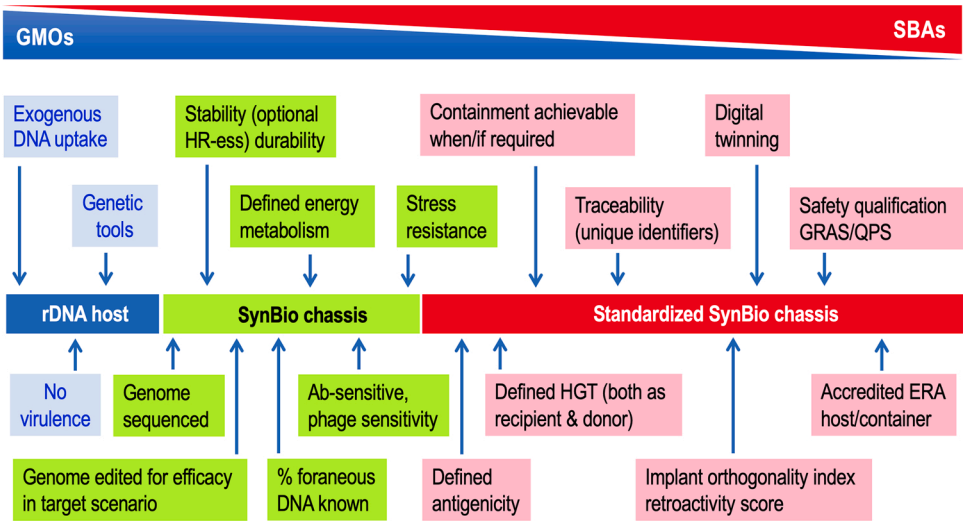

Fig. 1. The roadmap from environmental isolates to fully-fledged standardized SynBio chassis and from GMO (genetically modified organism) to SBA (SynBio agent). The scheme indicates the nature of the information that should be available for each category of strains. Note that there is not a defined boundary between GMOs and SBAs (see proposed definitions in Table 3). One important aspect of standardized chassis is their digital twinning that can be implemented through DNA barcoding as explained in the text. The final product of the process should be an effective and ERA-acceptable host of SynBio devices—or in general rDNA constructs.

important in the laboratory or in academic settings. Most of them deal with safety and efficacy issues, which need to be addressed to overcome environmental risk assessment (ERA) criteria (Table 2) and gain a green light by regulatory agencies. Properties of interest to this end include antigenicity and horizontal gene transfer (HGT) — either as donors or recipients of DNA. For some specific applications, containment of the strains themselves or at least barriers to HGT to/from them are necessary [25,26], while in others propagation of beneficial traits to the surrounding natural community might be desirable [,27], depending on the goal.

Table 1
A sample of proposed microbial chassis for SynBio[a].

Genus / species	Qualities of interest	References
Mycoplasma sp.	Small genome, vehicle for delivering therapeutic activities to the lung	[6]
Escherichia coli	Laboratory work horse, recombinant DNA host, abundant genetic tools	[49]
Pseudomonas putida	Tolerance to environmental insults (solvents, redox stress), platform for metabolic engineering	[14,50]
Bacillus subtilis	Laboratory workhorse, easy recombineering, efficient secretion systems	[11,15]
Corynebacterium sp.	Long time applications in industrial biotechnology, large-scale production of amino acids	[51,52]
Saccharomyces cerevisiae	Laboratory workhorse, easy genetic manipulations, optimal eukaryotic metabolic engineering platform	[52]
Pichia pastoris	Large-scale production of recombinant proteins & chemicals	[53]
Synechocystis/ Synechococcus	Photosynthetic organisms, CO_2 fixation, emerging metabolic engineering	[54,55]
Streptomyces sp.	Diverse secondary metabolism, production of antibiotics, efficient secretion systems	[56]
Vibrio natriegens	Super-rapid growth, easy to engineer, host of recombinant DNA constructs.	[57,58]
Lactobacillus sp	Platform for engineering in situ production of bioactives by designed probiotics	[59]
Alteromonas sp	Delivery of biodegradative and bioremediation activities to marine systems	[60]
Rhizobium sp.	Agents for targeting plan roots and designing new symbiotic systems	[61]
Yarrowia lipolytica	Biotransformations with apolar substrates and products	[62,63]
Halomonas sp	Growth at high densities in non-sterile seawater. Easy genetic manipulation	[64,65]

Note that many other chassis could be entertained, but in any case their number should be limited to a reasonable figure.

[a] Recapitulated and expanded from [66].

Table 2
Questions on risks borne by laboratory-designed microorganisms[a].

1. Can they colonize and eventually takeover natural microbial communities?
2. Is there a chance that they enter new niches that natural bacteria cannot?
3. Could they go into a stage of uncontrolled growth?
4. Can designed organisms trigger allergies or other undesirable immune reactions?
5. What are the chances of horizontal transfer of the synthetic genes to novel recipients?
6. Is there a tradeoff between safety and biotechnological efficacy of designed microorganisms?
7. Could engineered traits evolve towards virulence or other deleterious behaviour?
8. Are there performance scenarios capable of damaging life or property?
9. What is the environmental fate of synthetic genes?
10. Are there chances of malicious misuse?
11. Should they be endowed with traits for increasing their safety, traceability and predictability?

[a] Recapitulated and expanded from [67].

To bridge the gap between fundamental laboratory-based science and industrial applications, a standardised SynBio chassis should ideally be endowed also with a quantifiable portability score i.e. efficacy to express constructs assembled in other hosts. This would enable the design of biological circuits in a domesticated laboratory strain of reference and yet be able to operate the same genetic construct in a different chassis of another strain or species (e.g. one amenable to bioprocess upscaling). This should happen in order to avoid re-engineering at every step and minimising the need for a complete ERA reassessment. In either case, traceability of the designed strains with e.g. unique genome-born barcodes seems to be a must (see below). Not only for securing intellectual property of the constructs in question but also for accessing information useful to take countermeasures to propagation in case it is needed.

The scheme of Fig. 1 also embodies the difficulty in distinguishing SynBio chassis as something completely different from GMOs, as there is a clear continuum between the two. Nevertheless, in Table 3 we propose a nomenclature for the diverse terms that can be useful in classifying the various items into specific categories. In particular, we consider important the notion of synthetic biology agent (SBA, see above) as a standardized SynBio chassis genomically implanted with *cis/trans* genetic devices. Under this scheme, the distance between GMOs and SBAs is considerable (Fig. 1) and tagging specific biological objects as belonging to one class or the other for the sake of regulations should be feasible.

Table 3
Nomenclature for defining and distinguishing GMOs vs SBAs.

Term	Definition	Comments
Isolate	Naturally-occurring (micro) organism	Microbial strains with properties of either fundamental or applied interest
Variant	Naturally-occurring (micro) organism with defined changes in its native genome	Change(s) either spontaneously effected in an isolate or entered through rDNA technology
Recombinant DNA (rDNA)	DNA sequences from biological systems (either amplified from existing genetic material or altogether synthetic) propagated in designed vectors	This is the material for construction of genetic implants and devices thereof
rDNA host	Non-pathogenic variant capable of exogenous DNA capture and manipulable with genetic tools	Typical examples include the plethora of *E. coli* strains developed for DNA cloning
GMO (genetically modified organism)	rDNA host ± modified *trans/cis*-genic DNA	Strain modified to specifications with rDNA technology
Chassis	rDNA host optimized for deploying genetic devices under specified conditions	Biological frame where rDNA components can be plugged in and out for creating new functionalities
Genetic implant	Any synthetic DNA sequence added to the chassis' genome and encoding one or more biological parts	Implants typically involve a number of parts with a given relational logic for forming devices/circuits
SynBio construct	SynBio chassis + *cis/trans*-genetic implant(s)	The most frequent result of SynBio operations
Standardized chassis	Barcoded chassis that fulfils the 8 criteria of Fig. 1	Chassis that embodies a number of strict requirements for meeting industrial application standards
SBA (synthetic biology agent)	Standardized SynBio chassis + *cis/trans*-genetic implants/devices	Consolidated biological materials for biotechnological application (industrial, environmental)

Safe implementation of SynBio agents: standardized chassis should ease ERA certifications

It is not realistic to have an unlimited number of chassis. Instead, it will be more practical from an ERA point of view to define a reduced number of them for specific uses or environments that are thoroughly standardized, characterized and given a certain safety score. This would then limit ERA of specific agents (i.e. chassis + implants) to the effects of the new genes and their retroactivity with the genetic and physiological network of the host (see below). ERA issues of the different chassis must first gather information on [i] genomic sequence and its resilience/stability over time, i.e. built-in evolvability, [ii] efficacy in the target scenario, [iii] genetic stability (e.g. homologous recombination capacity, insertion sequences and other mutagenic elements) and durability, [iv] sensitivity to phages and antibiotics or other preventive tools for emergency clearance, [v] availability of advanced genetic tools, [vi] traceability, preferably engineered through genomic barcoding and linked to version control systems for strain engineering (see below), [vii] antigenicity, [viii] energy metabolism, [ix] stress resistance, [x] horizontal gene transfer capability (both as a donor and as a recipient) portability profile and [xi] environmental conditions for its persistence. All this should result in specific safety rankings, such as those that are currently in place for example for food related organisms, e.g. GRAS (generally regarded as safe in FDA nomenclature) or QPS (qualified presumption of safety in EFSA terminology), but associated with specific chassis and implants, not with a whole species.

Any SynBio construct is the result of combining a live chassis with one or more genetic additions. Emphasis on safety should thus be focused on the final agents and address the questions indicated in Table 2. Obviously, if we separate the questions into those applied to a pre-set standardized chassis and those to the new implants, we would considerably ease and accelerate the ERA process and the granting of permissions for widespread use. In that case, the questions would be limited to determining the retroactivity score/orthogonality index (i.e. the burden caused by the implant on the host physiology [28,29]), genetic stability (e.g. plasmid vs. chromosomal implant) and inspection of emergent properties. As sketched in Fig. 2, this is expected to make ERAs much simpler than those currently undergone by genetically engineered organisms, the studies of which have to be done on a case by case basis and on the whole organism rather than on specific parts of it. Moreover, a chassis with a portability appraisal would facilitate ERA even further, as the assessment of safety for any new genetic construct would need to be done only against the portability profile of the host and not the entire organism. Furthermore, adoption of standardized chassis will also enable application of similar or identical downstream processing protocols to many different industrial operations in which the only variable might be the specific pathway/genetic circuit implanted, in a fashion reminiscent of just replacing the software and the application in an electronic device, while keeping all the rest of the working pipeline.

And, if a different downstream process is required for faster/more economic acceleration, then a standardised chassis with a portability score would facilitate the transfer of pathways from one standardised chassis operating under one downstream process to another host that operates on a more efficient downstream process while keeping the functionality of the device intact. Chassis standardization thus eases both risk assessment and industrial appeal of SynBio-based practices, while at the same time optimizing the safety and control over synthetic biology agents. Here, the importance of barcoding as one key feature for differentiation of GMOs and SBAs must be emphasized.

Barcoding as an avenue to ease traceability and manage contingencies

There are many proposals for genetic firewalls to contain genetically engineered organisms and SynBio agents (see below). Current methods, however, do not allow detection of escape events occurring at frequencies below 10^{-12} [30], which is not enough to prove certainty of containment (CoC; [25]) for an environmental release. The scientific question about CoC is a very interesting one, but alas, achieving it is not yet in sight. We argue that barcoding can meet a considerable number of safety issues. Once decoded, barcodes can deliver the best available information for specific constructs (Fig. 3) such as their origins, parentage, safety, and modifications implemented in them [31] and serve as a complementary approach to any kind of containment measures. Although we argue here for the use of publicly retrievable

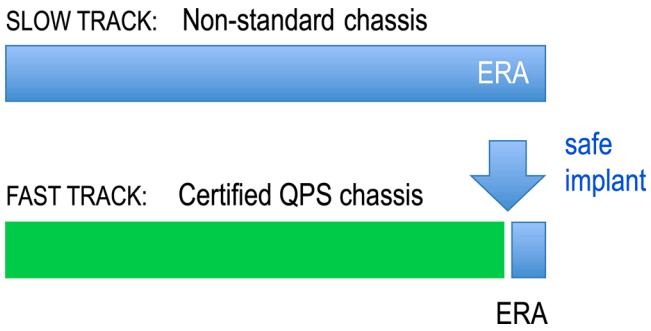

Fig. 2. SynBio chassis standardization eases the transit from laboratory scale to industrial and environmental applications in respect of regulations. ERA: Environmental Risk Assessment. For the fast track it is assumed that the QPS chassis has already been approved and that future examinations can rely on the previous assessment, thus only assessing the effect of the implant.

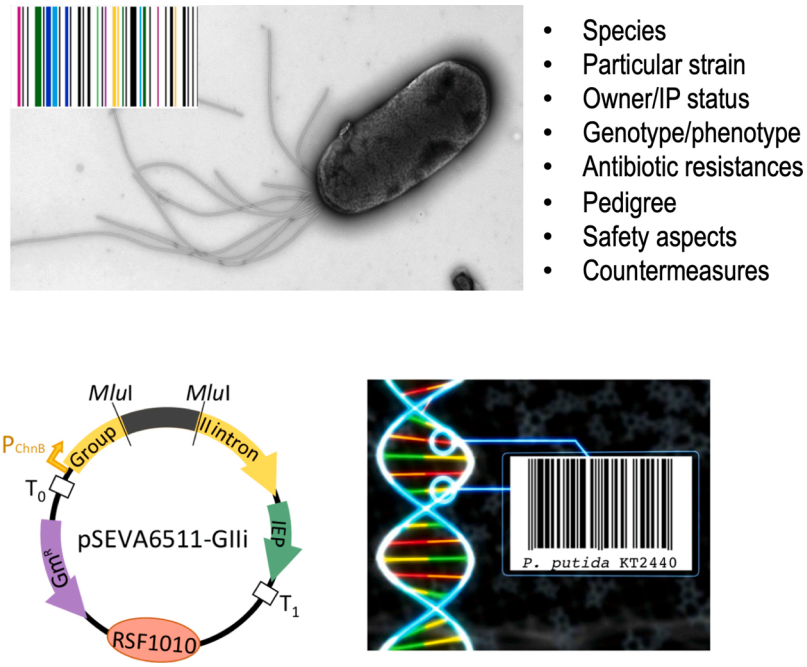

Fig. 3. Barcoding of SynBio chassis for traceability. Barcoding of SynBio chassis for traceability. Barcodes are unique numerical of graphical identifiers that allow instant recognition of given items and link them to their digital twins, where all information available becomes accessible. Computational and wet methods for genomic barcoding laboratory strains have been recently proposed [31]. Others based on the use of targetrons [23] or base editors [24] are being developed to mark the strains of interest (in this case *P. putida*) without relying on recombination. A specific construct for barcoding *Pseudomonas putida* with a targetron-based genetic tool is shown as an example. Subsequent improvements or derivatives of the same chassis could be then easily identified with updated barcodes that link the biological material to a digital twin i.e. web-based information-rich version control platform as proposed in reference [31]. Credit of the Figure: Elena Velazquez.

barcodes, the above rationale also applies to hidden barcodes, e.g. watermarks or steganographic data [71]. The latter, however, pose additional technological challenges. In any case, a barcode should be a *bio-orthogonal* [68–70] i.e., a DNA sequence that is devoid of biological significance. Thus, a bio-orthogonal barcode would have one or more of the following properties: [i] lack homologous sequences in the extant genomes, [ii] does not cause physiological burden, [iii] would not accumulate too many mutations (i.e. is not a mutation hot-spot), [iv] would persist under various stresses [31], [v] in the event of accidental barcode transcription, the barcode transcript will have weak or no secondary structure so it can be rapidly degraded by endogenous RNases. Identification of such DNA sequences requires a considerable computational effort, as the challenge lies more on the side of information technology than purely biological methods. Other approaches to barcoding could capitalize on the astronomic number of potential genetic codes (up to 4.8×10^{84} for 20 amino acids and a stop codon) where the altered genetic code itself would be the barcode [32]. Once such technicalities are solved, the rules for barcoding SynBio agents will have to be promulgated following agreed standards. Barcodes will not only make traceability simple, but it will also assign a non-ambiguous cipher to the increasingly improved versions of the same chassis (Fig. 3). This is the case with the operating systems (OSs) of computers as well as version control for updated variants of the same software. It could be possible to have a series of standardized chassis derived from the same original strain but barcoded to design version 1.0, 1.1, 2.0 etc. In this respect there is much to learn from the way computer industry has dealt in the past with similar challenges. In both cases, standardization and version control increase safety, enable tracing versions and sorts out IP issues, therefore easing regulatory frames. Ultimately, SynBio would benefit from adopting digital twinning technologies [68] which have had an enormous and positive impact on other industries.

The need of new ERA standards and metrics

While many ERA methodologies appear to be sufficient for assessment of potential risks of contemporary synthetic biology agents, it is necessary to ensure continued safety protection proportionate to risk, while at the same time enabling scientific, technological and socio-economic benefits. In a scenario where ERA would rely on current methods alone, allowing only the use of wild type comparators, and where synthetic biology is able to design and produce strains that diverge substantially from wild types, risk assessors will face considerable difficulties. In order to assess the cases presented, a long time and much effort will be needed to understand the level of change and potential impact of the engineered organisms.

One specific problem is the necessity to develop good metrics for ERA studies on SynBio agents. One metric currently available for engineered systems is the evaluation of the escape frequency for a given engineered containment circuit. Unfortunately—as mentioned above—the detection limit to assess the escape frequency is about 10^{-12}, but in order to be considered for a release into the environment, a physically contained industrial fermenter or even into a human patient, the proven escape frequency will have to be significantly lower. Decisions and regulations about how low should be informed by sound science, but the actual will ultimately be political. Moreover, there are no standards in terms of the media to test the escape frequencies in different environmental contexts (e.g. SCENIHR Report https://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_050.pdf). This state of affairs requires new standard operating procedures (SOPs) for quantifying risks and involving not only calculation of escape frequencies. They should also incorporate benchmarks and best practices available in what can be called *biosafety engineering* that addresses the issues out above. It is important to insist enough that there is a considerable need for ERA metrics beyond the mere calculation of escape frequencies. New techniques are badly needed for measuring the parameters specified above (Table 3) and answering the questions on afety of SynBio chassis and SBAs. The key challenge is testing their behaviour in a very large number of environmental conditions, not just those afforded by typical biology laboratories with flasks, plates, reactors and perhaps a number of micro and mesocosms. Fortunately, there is at this time a growing number of technical options for high-throughput testing of environmental conditions and simulation of physicochemical scenarios at a mm^3-scale. If we have organs or even human bodies on a chip, why cannot we develop environments on a chip? With these technologies in hand one could combine, in small droplets moving through a continuous tube and separated by gas and oil interfaces, a large number of parameters (humidity, O_2, textures, nutrients, chemical landscape, temperature, osmotic pressure, etc.) to produce thousands of different micro-

environments where SynBio agents at stake could be tested. Existing milli-fluidic platforms (e.g. the MilliDrop system www.millidrop.com) could be refactored/repurposed as experimental setups able to provide quantitative evidence on specific ERA questions on given constructs, supported with very solid statistics compared to existing methods. Operating such platforms over time—which could be seen as environmental equivalents to clinical tests for new drugs or tunnel facilities for the aviation industry — could deliver standards of reference for a wide range of situations, including long-term persistence, biological status of the cells at, scenarios and testing of possible countermeasures (e.g. antibiotics, phages).

Pseudomonas putida as an example of how to become a SynBio chassis

Because of their fast growth in the laboratory, their tolerance to solvents, the endurance to redox stress and the ease of genetic manipulations, strain *P. putida* KT2440 has emerged as a platform of choice for industrial biotransformations and even possible environmental release [33]. The relevant feature of this species is that *P. putida* metabolism gives priority to stress resistance as compared to building of biomass [34]. A key issue for deciding the advantage of a given strain as a host of recombinant constructs is that of stability and durability of engineered devices. In the best case, genetic implants typically compete with the host's resources for the endogenous gene expression machinery and the metabolic currency that fuels the process (i.e., retroactivity, see above [28,35]). In the worst case, the same implants may encode reactions that lead to toxic (by-)products (e.g. reactive oxygen species, ROS) that either directly damage the DNA or indirectly trigger a RNA polymerase, sigma S (RpoS)-mediated general stress response that drives cells into a mutagenic regime [36]. The nothing-out-of-the-ordinary result is the loss of the constructs involved. This stress response is shared by all potential bacterial chassis, but what are the specifics of *P. putida* under these circumstances? Two qualities of the strain KT2440 seem to be helpful to counteract these problems. One is the conspicuously suboptimal performance of the SOS scheme [37], which makes DNA damage less prone to drive cells into a general mutagenesis regime. Such a poor functioning of the RecA-based DNA repair and system could help to maintain the high degree of sequence homology of the *P. putida* genome (up to 30% of the whole genetic complement [38]). Moreover, these bacteria are less inclined to remove foreign DNA through recombination mechanisms. Another advantage of *P. putida* as a host of novel biochemical pathways, in particular those involving redox transactions, is that the endogenous NAD(P)H cofactor regeneration efficaciously quenches the direct effect of ROS on DNA through misincorporation of 8-oxoguanidine [39]. This mitigates the toxic effect of redox reactions causing havoc in the recipient. Note that both the diminished SOS system and the high rates of endogenous NAD(P)H regeneration are entirely native to the species and the strain. These features naturally ease the hosting of routes for biodegradation of organic, e.g. aromatic, chemicals, which characteristically start with dioxygenation of an aromatic ring, a strong redox reaction that is prone to generate ROS. It is noteworthy that the same dioxygenation of 2,4-dinitrotoluene (DNT) executed by the multicomponent enzyme DntA brings about a mutagenic regime when placed in *Burkholderia cepacia* [40] and *E. coli* [36] but has no apparent effect on *P. putida* KT2440 [39]. This changes, however, when NAD(P)H regeneration rates are artificially lowered, what results in a highly mutagenic status [39]. There is thus a clear connection between the core metabolic background of any given bacterial chassis and its ability to tolerate implantation of exogenous genetic devices — enzymatic or otherwise. Such a tolerance could be improved by adoption of additional genetic circuits that have been proposed for isolation of engineered implants [41]. A plausible downside of such over-engineering is that having systems with more components also causes cells to have more targets for potential mutations. For the time being these considerations are merely speculative, as comparative studies on long-term persistence of synthetic constructs are clearly missing — and badly needed.

Is *P. putida* safe?

A distinct matter that affects the consideration of *P. putida* as a reliable and usable chassis in large-scale applications is its biosafety level. Although many articles on the KT2440 strain of this species typically start with a statement about the GRAS status of the isolate, a careful appraisal of the supporting documents recently published [42] raises doubts not only about its safety score, but also about the taxonomy of one of the most used strains of the genus. Unlike what is generally believed, no *Pseudomonas* strains appear in the list of GRAS organisms or products [43] (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices). Instead, the KT2440 strain (together with some plasmids) belongs to the group of host-vector (HV) systems safety level 1, thus HV1. This rules that *P. putida* KT2440 can be handled in a P1 (or biosafety level 1, BSL1) facility with no special containment measures. Interestingly, a full-fledged GRAS status requires the microorganism to be harmless when ingested, a scenario that — to the best of our knowledge — has not been yet been documented for this bacterium. Note that the HV1 status is limited to strain KT2440, as some *P. putida* isolates are known as opportunistic human pathogens in hospitals [44]. The 1982 FDA certification thus documents that working with the KT2440 strain does not ask for specific precautions on methods and facilities for handling this microorganism in the laboratory. Yet, the community should cease to claim a formal GRAS status to the same microorganism, because it is (thus far) not classified as such. Note that a food substance could become GRAS either through scientific procedures or through experience based on long-term common use in food (https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras). Could the same experience-based criterion be applied to *P. putida* and other chassis-to-be?

As mentioned above, food and feed safety in Europe is accredited via EFSA through the QPS stamp. Upon a formal application procedure, if the assessment of a group of microorganisms concludes that they do not raise safety concerns, they can be granted an official QPS status [18]. Any microorganism that is assigned to that group does not need to undergo the full safety assessment. To be granted QPS status (https://www.efsa.europa.eu/en/topics/topic/qualified-presumption-safety-qps), a microorganism must meet the following criteria: [i] its taxonomic identity must be well defined, [ii] the available body of knowledge must be sufficient to establish its safety, [iii] the lack of pathogenic properties must be established and substantiated and [iv] its intended use must be clearly described. The specific issue of pathogenicity — one major aspect of ERA — might be problematic, as deletion of conspicuous virulence genes is necessary, but may not be sufficient, to reach an acceptable level of safety. The strain could re-acquire virulence genes via HGT and become hazardous again. Knowledge of the mechanisms that foster or impede gene flow to/from the species and strain at stake thus becomes of the essence, as additional features to prevent HGT might need to be engineered. Although this may not be straightforward to implement, the safest choice for containment of SBAs destined for release would be the adoption of a chassis with a different genetic code [45] or at least recoded in a few essential genes [46]. Obviously, safety levels should be adequate to the application scenario, which in the case of *P. putida* deals mostly with white (biotransformations) and brown (waste treatment) biotechnology. Note however that its prospective use for food and feed is not a fictional issue, as some research is underway to engineer *P. putida* KT2440 as a potential probiotic. We argue that KT2440 is safe to handle not only for the lack of noticeable virulence factors in its genome [47,48], but also by 40 years of extensive and intensive use by a large community without one single case of fortuitous infection reported — not unlike mutational breeding that is exempt from GMO status in Europe due to a long safety history. Moreover, the fact that other *P. putida* strains have caused rare opportunistic episodes is not

a strong argument to question the safety of KT2440. Contemporary synthetic biology is currently working on additional tools to further enhance biosafety, e.g. by changing the genetic code [5] or making viability dependent on a synthetic chemical (synthetic auxotrophies) which are feasible in *P. putida* should they be required by regulatory bodies or other environmental or health concerns.

Conclusion

The growing adoption of SynBio technologies, strains and products by the biotechnology industry has placed their unequivocal identification under the focus of regulatory agencies. SynBio jargon (including the term *chassis*) might be used in the academic realm with great flexibility, only limited by the tolerance of research journals to accept diverse types of neologisms and metaphors. But when the time comes to regulate the field in terms of permissions for industrial use, eality is that the boundaries between traditional GMOs and SynBio agents are quite blurry and objective criteria to distinguish them is difficult. In this article we propose some possible avenues to tackle the issue, but regulations ultimately boil down to numbers and thresholds that are arbitrarily set by the corresponding authorities (e.g. by the same token that they set tolerable levels of pollutants in water). One possible approach could involve quantification of the % of genomic DNA that has been inserted/deleted in the SynBio agent in respect to the ancestral host. Once such a level is agreed, the strain at stake would be a GMO if the % goes below the figure and an SBA when it is above the mark. But other criteria are equally possible or desirable: % or number of biological parts implanted, number of manipulative steps that were necessary to engineer the agent of even the share of new information implanted in the microorganism. On the basis of this preliminary score, one could then apply the battery of ERA tests discussed above. In any case, the incorporation of SBAs to the biotechnology industry of the future will demand a dramatic change in the way we run ERA from an individual assessment basis to focus on a limited number of well-accredited chassis along the lines herein presented.

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