ZKBS
The Central Committee on Biological Safety

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Recommendation by the German Central Committee on Biological Safety (ZKBS) on the risk assessment for

Mycoplasma mycoides JCVI-syn2.0 and *Mycoplasma mycoides* JCVI-syn3.0 as donor or recipient organism

according to § 5 paragraph 1 of the German Genetic Engineering Safety Regulations (GenTSV)

M. mycoides JCVI-syn2.0 and *M. mycoides* JCVI-syn3.0 are minimal organisms which were created at the J. Craig Venter Institute (JCVI) in 2016 [1]. They were derived from the synthetic strain *M. mycoides* JCVI-syn1.0 [2] which the ZKBS assigned to **risk group 2** in 2010 (Ref. no. 6790-05-01-94). The genome of *M. mycoides* JCVI-syn1.0 was synthetized *in vitro* and contains essentially the same genetic information as *M. mycoides* subsp. *capri* (**risk group 2**). *M. mycoides* subsp. *capri* is a well characterized Gram-positive bacterium belonging to the class of *Mollicutes* and is the pathogen which causes the contagious agalactia in goats [3]. Lethality is at 20 % or less, however, it might amount to up to 70 % in case of sepsis [4].

Mycoplasmas are adapted to the stable, nutrient-rich environment of their animal host. Typical virulence factors such as toxins, cytolysins or invasins are usually absent. The complex mechanisms of pathogenicity have not been completely elucidated for many mycoplasmas [5-7]. α-glycerophosphate oxidase has been described as a virulence factor of *M. mycoides* subsp. *mycoides* Small Colony. This enzyme, which is presumably also formed by *M. mycoides* subsp. *capri* [5], delivers significant amounts of H_2O_2, which is produced by the degradation of glycerin and damages the host cells. An ABC transporter for glycerin uptake, absent in less virulent strains, probably contributes to pathogenicity as well [6].

M. mycoides JCVI-syn3.0 was created to obtain an autonomously replicating cell which possesses only the genes absolutely necessary for survival under ideal conditions [1; 8]. Genes not essential for survival in complex media were identified using scientific literature and data derived from transposon mutagenesis experiments and were subsequently deleted. In general, the coding region, inclusive of the start and stop codon, was completely removed. The intergenic regions in gene clusters were also removed. Only the genetic elements which are needed for expression of further genes (e.g. ribosomal binding sites or promotors) were retained. The *in vitro* synthesis of the JCVI-syn3.0 genome proceeded in accordance with the Gibson Assembly® method [2]. The reduced genome was finally transferred to *M. capricolum* subsp. *capricolum* cells (**risk group 2**) and viable bacteria which only had this reduced genome were then selected with the aid of a selection marker [1].

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After three cycles, in which improvements to the eight segments were respectively made, **JCVI-syn2.0** was the result, a strain whose genome was smaller than that of all known naturally occurring bacteria (576 kb; 517 known genes: 478 protein-coding, 38 RNA-coding). **JCVI-syn3.0** was derived from JCVI-syn2.0 by removal of further 42 genes from the genome. The genome of 531 kb (473 known genes: 439 protein-coding, 35 RNA-coding) is only half as large as that of JCVI-syn1.0 (1079 kb; 901 known genes) [1]. Compared with the genome of JCVI-syn1.0, a total of 428 genes are missing, of which ~ 63 % have unknown functions, all 73 mobile elements and genes needed for the restriction of DNA as well as almost all lipoprotein genes. As the genome of JCVI-syn3.0 has been optimized for growth in full medium, it also lacks numerous genes for transport and metabolism, for example, most of the genes required for the utilization of other carbon sources than glucose. The 473 genes still remaining can be divided into four categories: Almost all genes needed for gene expression (195) and genome maintenance (34 genes) were retained. A large proportion of the genes (84) codes for constituents of the cell membrane (e.g. transporters for the uptake of small molecules like lipoproteins) or proteins of metabolism (81). 79 genes could not be allocated to any biological function. A basic function of some genes is known, whereas the function of 55 genes is completely unknown. Potential homologues of some of these genes are found in the genomes of various organisms. Presumably there are still undiscovered functions which are essential to life [1].

A recent publication by Jores *et al.* (2019) describes a bacterium derived from *M. mycoides* subsp. *capri* strain GM12 in the genome of which 68 genes not essential for growth were deleted [9]. Sixty-seven of these genes belong to the 437 genes deleted in JCVI-syn3.0 and code, among others, for components of the glycerol-dependent hydrogen peroxide metabolic pathway and for lipoproteins. Three of these genes that code for a hydrogen peroxide metabolic pathway are not deleted in JCVI-syn2.0 [6]. The growth rate of the strain *M. mycoides* GM12::YCpMmyc1.1-Δ68 described in Jores *et al.* is comparable to the parental strain. In animal experiments 10^9 colony forming units von *M. mycoides* GM12::YCpMmyc1.1-Δ68 were administered transtracheally in goats. Respectively eight goats obtained the wild type strain *M. mycoides* subsp. *capri* strain GM12 and eight goats the *M. mycoides* GM12::YCpMmyc1.1-Δ68. The animals infected with the wild type strain showed symptoms such as necrotising inflammations at the site of injection, fever and sepsis after only a short time and had to be killed within 5 to 6 days after the infection. The goats infected M. *mycoides* GM12::YCpMmyc1.1-Δ68 showed no clinical symptoms up to the end of the study (28 days). No pathological lesions, inflammations or necroses could be established upon euthanasia on day 28. In contrast to the goats infected with wild type strain, additionally no bacteria were able to be isolated from the blood of the goats infected with *M. mycoides* GM12::YCpMmyc1.1-Δ68.

Recommendation

Pursuant to § 5 paragraph 1 of the German Genetic Engineering Safety Regulation in conjunction with the criteria stated in Annex I of the GenTSV, *Mycoplasma mycoides* JCVI-syn2.0 is assigned **risk group 2** as a donor and recipient organism for genetic engineering operations, and *Mycoplasma mycoides* JCVI-syn3.0 **risk group 1**.

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Reasoning

The strains *M. mycoides* JCVI-syn2.0 and *M. mycoides* JCVI-syn3.0 are minimal organisms which have been derived from the strain JCVI-syn1.0. In comparison, their genome is reduced considerably, for which reason their growth is much slower.

It is obvious that genes that are required for the colonisation of a host and parasitic survival are no longer available in JCVI-syn2.0 and JCVI-syn3.0, because selection outside of the host took place and in full media . A comparison and a functional allocation of gene sequences of various mycoplasma, in particular those that participate in pathogenicity, however, is difficult. An analysis of the protein domains showed that 26 of the 479 *core* domains found in mycoplasma living in blood and tissues are missing in JCVI-syn3.0 [10].

Mycoplasma possess a number of potential virulence factors, whereby the loss of only one pathogenicity mechanism can lead to an attenuation. [6]. Thus, for example, the virulence of *M. mycoides*subsp. *mycoides* strains, the capsule polysaccharide biosynthesis of which is diminished, is greatly reduced although these strains still produce great quantities of H_2O_2 [6; 11]. The exact allocation and significance of genes participating in the pathogenicity of *M. mycoides* subspecies are, to date, not entirely understood. *M. mycoides* GM12::YCpMmyc1.1-Δ68, in which 67 genes that are also deleted in JCVI-syn3.0 are missing, showed itself to be entirely apathogenic in an animal experiment. As these genes are also deleted in JCVI-syn3.0 and JCVI-syn3.0, in comparison to JCVI-syn1.0, additionally features a clearly increased doubling time, JCVI-syn3.0 is assigned to **risk group 1**

In JCVI-syn2.0, 42 of the genes deleted in JCVI-syn3.0 are still present, as amongst them the genes *glpF, glpK* and *glpO*, which are also deleted in GM12::YCpMmyc1.1-Δ68. The products of these genes, as a bypass pathway, make possible the import, the phosphorylation, and the oxidation of glycerol, which allows for the release of toxic H_2O_2 in the host cell [7]. Normally the import of glycerol take place via an ABC-transporter, the operon *gtsABC* of which is deleted in JCVI-syn2.0 and also in JCVI-syn3.0. Because it is not clear whether the remaining genes for glycerol transport represent virulent factors and, until now, no experimental data on the pathogenicity of JCVI-syn2.0 are available, JCVI-syn2.0 will continue to be assigned to **risk group 2**.

Literature

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BVL_FO_05_4100_402_V1.5