ZKBS
The Central Committee on Biological Safety

Az. 45270_a April 2021

# Statement of the ZKBS on the suitability of *Escherichia coli* K12-derived strains as part of biological safety measures according to § 8 para. 1 GenTSV

## 1. General Information

With the entry into force of the amendment to the Genetic Engineering Safety Regulation (GenTSV) in March 2021, it is necessary that, in accordance with § 7 para. 5 GenTSV, the continued existence of already recognised biological safety measures (here: vector and recipient systems) is confirmed by the Central Committee on Biological Safety. Section 8 para. 1 of the amended GenTSV specifies the conditions under which the classification of a recipient organism can be recognised as part of a biological safety measure. These conditions are met if 1. a scientific description and a taxonomic classification of the recipient organism are available, 2. the recipient organism is not pathogenic for humans, animals or plants and does not exhibit any environmentally hazardous properties, 3. the recipient organism can only be multiplied under conditions that are rarely or not at all encountered outside genetic engineering facilities and 4. the recipient organism engages in only minimal horizontal gene exchange with other species.

This statement examines and evaluates whether strains derived from the original *Escherichia coli* K-12 isolate (*E. coli* K12-derived strains) meet the abovementioned conditions.

The *E. coli* K12-derived strains were already recognised as a biological safety measure (recipient organism) in the "Guidelines for protection against hazards from *in vitro* recombinant nucleic acids" in force since 1978 (most recently in the 5th revised version of 1986). This was also continued in the 1st Genetic Engineering Act of 1990. In the decades of widespread use of *E. coli* K12-derived strains as a biological safety measure, they have proven to be safe without exception.

### 1.1 Scientific description

The species *E. coli* belongs to the *Enterobacteriaceae* family. The family includes Gram-negative, non-sporulating, chemoorganotrophic, facultative anaerobic rods. *E. coli* belongs to the microbiome of the large intestine of almost all warm-blooded mammals. Some pathogenic members of the species can cause severe diseases in humans and animals [1].

*E. coli* K-12 is a strain isolated from a stool sample of a convalescent diphtheria patient in 1922. The strain was included into the collection of the Bacteriology Department of Stanford University in 1925 under the designation "K-12" [2]. Derivatives of the *E. coli* K-12 strain have been used for genetic studies since the 1940s. The original K-12 strain is lambda-lysogenic and contains the F factor. The phage lambda was later removed by UV radiation and the F factor by segregation [3]. Today's *E. coli* K12-derived strains contain neither the phage lambda nor the F factor unless these have been subsequently reintroduced. For 100 years, *E. coli* K12 and K12-derived strains have been cultivated exclusively outside the natural

habitat. For about 70 years, *E. coli* K12 and K12-derived strains were kept exclusively on agar plates or in stab cultures before the use of cryopreservation became established. Therefore, the bacteria are adapted to existence in complex media. As a result of permanent cultivation, spontaneous mutations arose in the strains [4]. For instance, they had lost K- and O-antigens after 30 years of cultivation [5, 6]. The spontaneous and introduced mutations of *E. coli* K12-derived strains are diverse and well characterised, making the strains versatile in basic research and biotechnology. So for instance, mutants with auxotrophies contribute to low survival in the environment and strains with a defect in the *recA* gene show a marked reduction in DNA repair mechanisms and recombination ability of the bacterial genome. The genomes of several *E. coli* K12-derived strains are completely sequenced [7–9].

*E. coli* K12 and its diverse derivatives are scientifically very well characterised model organisms with a taxonomically clear classification.

## 1.2 Pathogenic potential of *E. coli* K12 strains

Pathogenic *E. coli* strains are primarily associated with gastrointestinal disease symptoms. In the *E. coli* K12 strain, most virulence factor genes are absent that are present in intestinal and extraintestinal pathogenic *E. coli* strains (IPEC and ExPEC) or also individually or in combination in apathogenic isolates (ECOR strains). In pathogenic *E. coli* strains, virulence factor genes are usually arranged in the form of pathogenicity islands in large blocks. These can be located in the chromosome, on plasmids, or on the genome of bacteriophages. Corresponding chromosome segments, plasmids or prophages are not present in *E. coli* K12 [10]. Genes for most adhesins as well as for toxins, O-antigens, glycocalyx proteins, invasins and other virulence factors are missing [10, 11]. *E. coli* K12 is unable to attach to the mucosa of the intestinal epithelium since no adhesins for this purpose are expressed, the production of O-antigens is impaired, and no capsule is formed. If *E. coli* K12 derivatives were accidentally ingested orally by laboratory personnel or accidentally entered its bloodstream, then they would not establish themselves or would be rapidly recognised and eliminated by the body's own immune system [11]. A long residence time or a permanent colonisation of humans and animals with *E. coli* K12 is therefore excluded. Symptoms of disease following oral ingestion of *E. coli* K12-derived strains have not occurred in volunteers or in laboratory animals [12–14]. No health hazard is observed in the mouse model after peritoneal injection of $10^6$ colony-forming units of *E. coli* K12 derivatives. Seven days after injection, no living bacteria were detected in the organs [12]. For *E. coli* wild-type strains from patient blood samples, the median lethal dose when administered intravenously in the mouse model is $10^5$ live bacteria. It takes $10^3$ -$10^4$ times more bacteria for *E. coli* K12-derived strains to achieve a mortality of 30 - 40 % [15]. An accidental injection of a relevant quantity of *E. coli* K12 derivatives that is hazardous to health is not to be expected in genetic engineering work, so that there is no hazard potential.

Certain pathogenic *E. coli* strains are able to multiply outside the normal host. For example, the EHEC strain *E. coli* O157:H17 multiplies on irrigated and/or damaged plants [16, 17]. However, this or other *E. coli* strains do not cause plant diseases [18–20]. Likewise, *E. coli* K12-derived strains do not show pathogenicity to plants [21].

*E. coli* K12 and K12-derived strains are apathogenic and thus pose no risk to humans, animals, or plants.



## 1.3 Multiplication ability of *E. coli* K12 outside genetic engineering facilities

The survivability of *E. coli* K12-derived strains in the intestine has been tested in numerous studies in humans and animals. At the latest 6 days after oral administration, *E. coli* K12-derived strains are no longer detectable in the human intestine [13, 14, 22, 23]. In mice, *E. coli*

K12-derived strains also do not establish permanently in the intestine [24]. It is also not possible for *E. coli* K12-derived strains to colonise the intestines of germ-free rats [25].

Multiple studies with soils, surfaces and water samples further show that *E. coli* K12-derived strains cannot survive in the environment either. In nonsterile soils, *E. coli* K12-derived strains did not survive for more than 28 days [21, 26–28]. Wild-type *E. coli*, on the other hand, survive in soil until the end of an 80-day study period [29]. In the event of a release of *E. coli* K12-derived strains as aerosols, a survival time of up to a maximum of 2 hours in air can be assumed [30]. Depending on the material, sedimented bacteria survive on surfaces for up to 20 days (wooden surfaces), but on average no longer than 24 hours [30]. When *E. coli* K12-derived strains are distributed on lettuce plants through sprinkler systems, no *E. coli* can be detected after a maximum of 7 days [21]. Pathogenic *E. coli* wild types, on the other hand, multiply on lettuce plants and are still detectable until the end of an 30-day study period [17]. It is assumed that *E. coli* K12-derived strains cannot attach to the plant surface since they do not express curli fimbriae [31]. If the release of *E. coli* K12-derived strains takes place in lotic ecosystems, the survival time is a maximum of 15 days. When the release takes place in streams near the inlet of treated wastewater, the bacteria survive for a maximum of 10 days [28, 32, 33]. When released into lentic ecosystems, *E. coli* K12-derived strains survive up to 16 days. If culture media are also discharged into the water body, the bacteria can be detected in the water body for up to 10 weeks [34]. In marine ecosystems, *E. coli* K12-derived strains survive up to 6 days at 15 °C water temperature [35]. In general, the survival time of the bacteria decreases with increasing water temperature in both salt and fresh water [32, 35]. The presence of *E. coli* in water does not lead to colonisation of the gastrointestinal tract of fish [36].

In summary, the data show that *E. coli* K12-derived strains can only survive for a short time in soils, in aerosols, on surfaces, on plants as well as in water bodies. Permanent establishment in the environment does not occur.

### 1.4 Horizontal gene transfer from *E. coli* K12-derived strains to other organisms

Horizontal gene transfer in bacteria can occur through processes such as transformation, transduction, conjugation, and mobilisation of genetic material. These molecular mechanisms have been extensively studied [37]. The frequency of horizontal gene transfer events from *E. coli* K12-derived strains to endemic bacterial species has been studied in numerous *in vitro* experiments. Rare gene transfer from *E. coli* to eukaryotic cells has so far only been shown in special laboratory experiments and does not represent a natural process [38].

#### 1.4.1 Transformation

*E. coli* K12, like *E. coli* in general, is not able to actively take up DNA from the environment and thus does not exhibit a natural competence that is present in numerous other bacterial species [39]. DNA only enters *E. coli* cells under experimental conditions that make the outer and inner membrane of the bacteria permeable, e.g., by using physical (electroporation) or chemical ($Ca^{2+}$ shock) methods. While the natural habitat of *E. coli* may contain other bacterial species with a natural competence for DNA uptake, it is improbable that free DNA from dead *E. coli* K12 cells will be taken up by such bacterial species in the intestine, as DNA is rapidly degraded there [39, 40].

### 1.4.2 Transduction

Various transducing phages have been used in genetic studies of *E. coli* K12-derived strains. Of particular importance are the phages lambda and P1.

The temperate phage lambda was already present in the original *E. coli* K-12 isolate. During the lysogenic cycle, the integration of the lambda DNA occurs at a specific site into the host DNA. After induction, in rare cases, the excision of the prophage can be imprecisely, so that genes adjacent to the integration site (*bio*- or *gal*-operon) are integrated in the genome of the phage. Such phage particles are transducing and transfer *bio*-genes or *gal*-genes. Thus, the prophage lambda belongs to the special transducing phages that only transfer specific bacterial DNA segments [41]. No transducing phage particles are formed during the lytic cycle when a host is infected with lambda. The phage lambda has a very narrow host range. Despite the fact that the lambda receptor maltoporin is also present in other Gram-negative bacterial species, only *E. coli* strains are infected due to the accessibility of the receptor through the LPS composition [42]. The lambda phage is thus not relevant for horizontal gene transfer events to other bacterial species.

Of greater importance for horizontal gene transfer is the temperate phage P1, which is characterised by a broad host range. Besides *E. coli*, phage P1 can infect *Shigella dysenteriae*, *Klebsiella aerogenes*, *Citrobacter freundii*, *Erwinia amylovora*, *Proteus mirabilis* and other bacterial species [43]. After a lytic multiplication cycle, 0.3 - 0.5 % of P1 phage particles are transducing. The production of transducing particles is not coupled to the induction of a prophage. Transducing phage particles contain only DNA of bacterial origin (chromosome or plasmid DNA). Phage P1 is one of the generally transducing phages and transfers DNA segments of approx. 100 kbp.

### 1.4.3 Conjugation and mobilisation

Bacterial conjugation is a process starting with a physical cell contact between donor and recipient and a subsequent transfer of a DNA single strand (ssDNA). In contrast to the recipient, the donor carries a conjugative plasmid. Conjugative plasmids have genes and sequences that are essential for DNA transfer (*mob, tra, oriT*). An example of a conjugative plasmid is the F factor. The *tra* operon of the F factor encodes the proteins involved in conjugation. Other conjugative plasmids have homologues of the *tra* genes. With the help of the F pilus, direct cell-cell contact is established between donor ($F^+$) and recipient ($F^-$) and, after retraction of the pilus, a stable conjugation pair ("mating pair") is produced. Subsequently, the relaxase TraI generates a single-strand break at the origin of the transfer (*oriT*) of the F factor. The TraI relaxase then unwinds the dsDNA. The TraD protein ("Coupling Protein") actively transports the now linear ssDNA of the F factor through a complex pore consisting of Tra proteins into the cytoplasm of the recipient (typeIV secretion, [44]). Meanwhile, rolling circle replication of the F factor takes place in the donor. In the recipient, the ssDNA recirculates and the complementary DNA strand is synthesised [45]. F plasmids with a mutation in *traD* are no longer conjugative but can still mobilise mobilisable plasmids with their own gene for a "coupling protein".



Non-conjugative plasmids that have an *oriT* and mobilisation genes (*mob*) can be transferred in the presence of a conjugative helper plasmid. However, not every mobilisable plasmid is mobilised by every conjugative plasmid. The ColE1 plasmid found in *E. coli* is a representative of plasmids that can be mobilised by the F factor and plays a major role in molecular biology. The *mob* gene products form a relaxation complex and the ssDNA of the ColE1 plasmid can now be transferred to a recipient by the transfer machinery of the F factor.

In addition to the described cases of conjugation, in which conjugative or mobilisable plasmids are transferred to recipients, chromosomal DNA can also be transferred by conjugation if conjugative plasmids are integrated in the chromosome of the donor. In the case of the F factor, integration into the host chromosome occurs via recombination between insertion elements (IS elements) of the F factor and those in the host chromosome. After integration, the F factor no longer controls its own replication. This does not affect the function of the *tra* operon. If a donor with integrated F factor meets an $F^-$ recipient, conjugation is triggered as in an $F^+$ cell and DNA transfer is initiated at the *oriT*. Since the F factor is now part of the chromosome, chromosomal genes can also be transferred after the transfer of F factor DNA. Through homologous recombination, these can be integrated into the chromosome in the recipient. Such strains are called high frequency of recombination (Hfr) strains. Sometimes integrated F factors can be excised from the chromosome. In individual cases, the excision proceeds imprecisely due to illegitimate recombination events, so that chromosomal genes are included in the released F factor. Such a strain is called F-prime (F') [46]. The integration of the F factor into the chromosome as well as the excision are RecA-dependent. By choosing *E. coli* K12-derived strains with a mutated *recA* gene, the spontaneous formation of Hfr and F' strains can be significantly reduced in genetic engineering with the F factor [47]. Other conjugative plasmids, such as the IncP plasmid RP4, may also be present chromosomally integrated, either through naturally occurring integration processes or as a consequence of genetic engineering.

Mobilisation of a plasmid without *mob* and *oriT* is also possible if a cointegrate is formed in the donor cell by recombination between the non-mobilisable and the conjugating plasmid. This chimeric plasmid can be transferred to a recipient by conjugation. Here, the cointegrate can be resolved by recombination [48, 49].

Conjugative plasmids with a broad host range are of significance for horizontal gene transfer by conjugation. These plasmids can induce contact with cells of different species. Furthermore, they are able to replicate in recipients of other species and escape their modification and restriction system for the elimination of foreign DNA [50]. The IncP plasmid RP4 is an important representative of conjugative plasmids with a broad host range and the ability to mobilise. It can replicate in almost all alpha, beta and gamma proteobacteria [50, 51]. Because of this property, it is used in genetic engineering to mobilise plasmids with an *oriT* to various Gram-negative bacterial species. The F factor, on the other hand, is a plasmid with a narrow host range that can only replicate in some species of the *Enterobacteriaceae* family. It is therefore of little importance for horizontal gene transfer [52, 53].

### 1.4.4 Reported horizontal gene transfer events

The frequency of horizontal gene transfer events from *E. coli* K12-derived strains to endemic bacterial species was investigated in different environments. No transfer of conjugative plasmids from *E. coli* K12-derived strains to other bacterial species in the gut has been documented [54, 55]. In comparison, wild-type *E. coli* strains are able to transfer conjugative resistance plasmids in the animal gut to *Salmonella enterica* subsp. *enterica* ser. Typhimurium [56].



Furthermore, the horizontal gene transfer of *E. coli* K12-derived strains in soil was investigated. A transfer of a conjugative RP4 derivative from *E. coli* K12-derived strains to *Sinorhizobium fredii* could only be observed at high cell densities of $10^6$ to $10^9$ donor and recipient cells per g sterile soil [57]. In nonsterile soil, transfer of RP4 by *E. coli* K12-derived strains to the endemic Gram-negative bacteria *Pseudomonas putida, Burkholderia cepacia* and *Pseudomonas fluorescens* could be detected [58]. Mobilisation of non-conjugative plasmids in *E. coli* K12-derived strains to other soil bacteria has not been observed [59]. In sterile seawater, transfer

of RP4 from *E. coli* K12-derived strains to *Pseudomonas fluorescens, Aeromonas hydrophila, Pseudomonas cepacia* und *Enterobacter cloacae* was observed [60].

## 2. Recommendation

According to § 8 para. 1 GenTSV, *Escherichia coli* K12-derived strains are recognised as part of a biosafety measure in which neither conjugative plasmids with broad host range and ability to mobilise nor generally transducing prophages with broad host range are present.

## 3. Reasoning

*E. coli* K12-derived strains fulfil the prerequisites of § 8 GenTSV for recognition as recipient organisms for biological safety measures. They are scientifically very well described. Neither are *E. coli* K12-derived strains pathogenic for humans, animals or plants, nor can the bacteria multiply or become permanently established in the gut or on plants. The survival of *E. coli* K12-derived strains outside of genetically engineered facilities has been well studied and it has been shown that the bacteria can only survive for short periods of time in soils, aerosols, surfaces, and waters. *E. coli* K12-derived strains do not pose a risk to the legal assets according to § 1 para. 1 GenTG due to their low survivability in the environment and their apathogenicity. Horizontal gene transfer from *E. coli* K12-derived strains to bacteria in humans and in the environment is generally very low.

However, the very low gene transfer potential does not apply to those *E. coli* K12-derived strains in which conjugative plasmids with a broad host range and ability to mobilise, and generally transducing prophages with a broad host range, are present. Thus, the possibility of horizontal gene transfer for these *E. coli* K12 variants is no longer considered to be low. Recipient strains with e.g. RP4 plasmid and/or P1 prophages are thus not recognised as a biological safety measure.

Information on whether individual *E. coli* K12-derived strains are suitable as biosafety recipient strains according to the criteria set out in this statement will be collected and made available in the biosafety recipient strain database maintained by the ZKBS administrative office.

## Literature


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