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Position Statement of the ZKBS on the suitability of *Pseudomonas alloputida* KT2440 (formerly *Pseudomonas putida* KT2440) 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, paragraph 1 of the amended GenTSV specifies the conditions under which the use of a recipient organism can be recognised as part of a biological safety measure. These are fulfilled if 1. a scientific description and a taxonomic classification of the recipient organism are available, 2. the propagation of the recipient organism is only possible under conditions that are rarely or not encountered outside genetic engineering facilities, 3. the recipient organism is not pathogenic for humans, animals or plants and does not have any environmentally hazardous properties and 4. the recipient organism only engages in minor horizontal gene exchange with other species.

This Position Statement examines and evaluates whether *Pseudomonas alloputida* KT2440 (formerly *Pseudomonas putida* KT2440) fulfils the abovementioned conditions.

P. alloputida KT2440 was recognised as a suitable recipient organism for biological safety measures as early as 1990, when the GenTSV came into force. In the decades of widespread use of *P. alloputida* KT2440 and its derived strains as biological safety measures, they have proven to be safe without exception.

1.1. Scientific description

The species *P. alloputida* belongs to the family *Pseudomonadaceae*. The family includes Gram-negative, non-sporulating, chemoorganotrophic, aerobic rods [1]. *P. alloputida* is a worldwide saprophytic inhabitant of soils, especially the rhizosphere of plants [2]. Some pathogenic members of the species can cause diseases in humans and animals [3].

P. alloputida KT2440 is a derivative of the strain *P. alloputida* mt-2, which was isolated from the soil of a vegetable garden in Japan in 1960 [4]. The *P. alloputida* KT2440 strain was established in the laboratory of Kenneth Timmis in 1981 and is diverged from *P. alloputida* mt-2 by the spontaneous loss of the TOL plasmid pWW0 [5]. Both strains have been cultivated

exclusively outside the natural habitat since their establishment. Until 2019, *P. alloputida* mt-2 and KT2440 were assigned to the species *Pseudomonas putida* [6]. The genome of *P. alloputida* KT2440 has been completely sequenced several times within the last 20 years [7, 8]. Due to this, it could be shown that spontaneous mutations occur in the strain as a result of permanent cultivation [7]. Four chromosomally integrated prophages are present in its genome, but they are not capable of generating infectious phage particles [9]. The strain *P. alloputida* KT2440 is well characterised and has many uses in biotechnology.

P. alloputida KT2440 is a scientifically very well characterised model organism with a taxonomically clear classification.

1.2. Pathogenic potential of *P. alloputida* KT2440

In the past, *P. alloputida* has rarely appeared as an opportunistic human pathogen. Diseases with *P. alloputida* were mostly catheter-associated bacteraemia and urinary tract infections [3]. Therefore, *P. alloputida* was assigned to risk group 2 according to § 6 in conjunction with § 5 para. 1 GenTSV (Ref. 6790-05-01-099; updated February 2023).

Most virulence factor genes present in pathogenic *P. alloputida* isolates or in the pathogenic closely related species *P. aeruginosa* are absent in *P. alloputida* KT2440 [8, 10, 11]. These include genes for the expression of exotoxins, specific hydrolytic enzymes, type III secretion systems, O-antigen synthesis enzymes and other virulence factors [8, 12] The pathogenic potential of *P. alloputida* KT2440 has been investigated in animal models. Thus, in the mouse model, the intraperitoneal injection of 10^9 colony-forming units (CFU) of *P. alloputida* KT2440 is not harmful [13]. Inoculation of healthy and damaged skin of rats with 10^5 CFU of *P. alloputida* KT2440 had no adverse effects. Pathogenic *P. alloputida* isolates, on the other hand, were capable of severely damaging the skin at the same inoculation dose [14]. There are no reports of infections with *P. alloputida* KT2440 in animals and humans. The strain *P. alloputida* KT2440 is assigned to risk group 1.

Under laboratory and greenhouse conditions, *P. alloputida* KT2440 is able to colonise the roots of many plants [15, 16]. Inoculation of the roots of maize, soybean and pepper plants with *P. alloputida* KT2440 resulted in activation of systemic resistance to foliar disease pathogens and promoted root growth [13, 15, 17]. Phytopathogenic effects have not been described for *P. alloputida* KT2440.

P. alloputida KT2440 is apathogenic and without hazard potential for humans, animals and plants.

1.3. Multiplication ability of *P. alloputida* KT2440 outside genetic engineering facilities

Multiple studies with soil and water samples show that *P. alloputida* KT2440 cannot propagate outside genetic engineering facilities. In field trials, *P. alloputida* KT2440 was no longer detectable in soils at an inoculation level of 10^7 CFU per g soil after 50 days [19, 21]. In cultivated soils, *P. alloputida* KT2440 persisted in the rhizosphere until the end of the experimental period of 60 days [16, 18]. Bacterial density decreased only slightly during this period. Survival in water was investigated with the parent strain *P. alloputida* mt-2. In non-sterile freshwater samples, bacterial density decreased by a factor of hundred within 10 days and by a factor of thousand by the end of the experimental period of 70 days [19]. In

wastewater, the bacterial density of *P. alloputida* KT2440 was reduced by a factor of ten thousand within 55 days [20].

In summary, this shows that there is no evidence of propagation of *P. alloputida* KT2440 in the environment.

1.4. Horizontal gene transfer from *P. alloputida* KT2440 to other organisms

Horizontal gene transfer in bacteria can occur through processes such as transformation, transduction, conjugation and mobilisation of genetic material, the molecular processes of which have been extensively studied [21].

In contrast to various other bacterial species, *P. alloputida* KT2440, like *P. alloputida* in general, is not able to actively take up DNA from the environment and thus does not exhibit natural competence for transformation with free DNA [22, 23]. DNA only enters *P. alloputida* cells under experimental conditions that make the outer and inner membrane of the bacteria permeable for this purpose, e.g. by using physical methods (electroporation) [24].

Generalized transducing phages with a broad host range largely contribute to horizontal gene transfer in bacteria. Such phages can occasionally package DNA of bacterial origin only (chromosomal or plasmid DNA) into infectious phage particles and infect hosts from multiple bacterial families. However, no phages are known to date that can infect *P. alloputida* KT2440 [9]. Of the four prophages of *P. alloputida* KT2440, two can be excised from the chromosome after prophage-inducing treatments. However, in no case does the formation of infectious phage particles occur [9].

Another process of horizontal gene transfer is the transfer of plasmids by conjugation. Bacterial conjugation is a process in which physical contact occurs between donor and recipient cells, followed by the transfer of a DNA single strand. Strain *P. alloputida* mt-2 contains the conjugative TOL plasmid pWW0, which can replicate in *Escherichia coli, Erwinia chrysanthemi, Hydrogenophaga palleronii, Serratia* spp. and *Burkholderia* spp. and thus has a broad host range [25]. However, strain *P. alloputida* KT2440 has lost this plasmid and does not contain any other plasmids [8], so that gene transfer using conjugation including mobilisation is excluded.

2. Recommendation

According to § 8 para. 1 GenTSV, *P. alloputida* KT2440 and its 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

P. alloputida KT2440 fulfils the requirements of § 8 para. 1 GenTSV for recognition as a recipient organism for biological safety measures. The strain is scientifically very well described and apathogenic for humans, animals and plants. *P. alloputida* KT2440 therefore does not pose a danger to legal interests according to § 1 Para. 1 GenTG. The survival of *P. alloputida* KT2440 outside genetic engineering facilities has been well studied and it has been shown that *P. alloputida* KT2440 does not spread in soils and waters, but persists in the

rhizosphere of plants. Horizontal gene transfer from *P. alloputida* KT2440 to other bacteria is not expected.

Information on whether individual *P. alloputida* KT2440 derived strains are suitable recipient strains for biological safety measures according to the criteria set out in this Position Statement will be collected and made available in the database of recipient strains for biological safety measures maintained by the ZKBS administrative office.

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