Identification of strains *Bacillus aerophilus* MTCC 7304\(^T\) as *Bacillus altitudinis* and *Bacillus stratosphericus* MTCC 7305\(^T\) as a *Proteus* sp. and the status of the species *Bacillus aerius* Shivaji et al. 2006. Request for an Opinion

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On the basis of 16S rRNA, *rpoB*, *gyrB* and *pycA* gene sequence analyses, characterization of biochemical features and other phenotypic traits and pulsed-field gel electrophoresis (PFGE) fingerprinting, it was ascertained that strains *Bacillus aerius* MTCC 7303\(^T\), *Bacillus aerophilus* MTCC 7304\(^T\) and *Bacillus stratosphericus* MTCC 7305\(^T\) do not conform to the descriptions of the type strains of the respective species. Strains MTCC 7303\(^T\) and MTCC 7304\(^T\) were indistinguishable from *Bacillus altitudinis* DSM 21631\(^T\), while strain MTCC 7305\(^T\) should be classified as a representative of a *Proteus* sp. Our attempts to find other deposits of the type strains of these species were unsuccessful. Therefore, the results support the Request for an Opinion on the status of the species *Bacillus aerophilus* and *Bacillus stratosphericus* by Branquinho et al. [Branquinho, R., Klein, G., Kämpfer, P. & Peixe, L. V. (2015). Int J Syst Evol Microbiol 65, 1101]. It is also proposed that the Judicial Commission should place the name *Bacillus aerius* on the list of rejected names if a suitable replacement type strain cannot be found or a neotype is not proposed within two years following the publication of this Request (Rule 18c).

Four novel bacterial species, *Bacillus aerius*, *Bacillus aerophilus*, *Bacillus stratosphericus* and *Bacillus altitudinis* were described by Shivaji et al. (2006) on the basis of a polyphasic study of four strains, 24K\(^T\), 28K\(^1\), 41KF2a\(^T\) and 41KF2b\(^T\), respectively. Before publication of the species descriptions, their type strains were deposited at the microbial collections of India (MTCC) and Japan (JCM).

*B. aerius*, *B. aerophilus* and *B. stratosphericus* were described as phylogenetically close to *Bacillus licheniformis* and *Bacillus sonorensis* while *B. altitudinis* was in a *Bacillus pumilus* clade of a neighbour-joining tree based on 16S rRNA gene sequences (Shivaji et al., 2006). However, when in 2009 we analysed the 16S rRNA gene sequences submitted to the databases by S. Shivaji, a discrepancy between the published data and the results obtained was found. *B. aerophilus* and *B. stratosphericus* were also neighbours of *B. pumilus* in phylogenetic trees. This was a result of the sequences analysed being new versions of the *B. aerophilus* and *B. stratosphericus* sequences. The new versions, which were submitted to GenBank in 2008, after the article was published, had only 95.0 % and 95.8 %
Similarities to the original versions used in the species descriptions.

To find a reason for the discrepancy, efforts to acquire the strains were made, but the type strains of *B. aerius*, *B. aerophilus* and *B. stratosphericus* were not available. They were absent in the JCM catalogue. The strains were listed in the MTCC catalogue, but they were also not available; according to a response by the MTCC curator to the request for strains, sending these strains abroad was not permitted. The search for other sources of strains, including from the strain depositor, was unsuccessful as well, and notes on a possible loss of the species type strains were added to the information on these species on the LPSN website (http://www.bacterio.net/bacillus.html). Attempts to obtain the *B. aerophilus* and *B. stratosphericus* type strains were made once again during description of the novel species *Bacillus xiamenensis* but also failed (Lai et al., 2014).

Only recently, we were able to obtain *B. aerius* MTCC 7303<sup>T</sup>, *B. aerophilus* MTCC 7304<sup>T</sup> and *B. stratosphericus* MTCC 7305<sup>T</sup> from a coauthor of this article working in India. *B. altitudinis* DSM 21631<sup>T</sup> was obtained from the DSMZ. Growth of the bacteria and study of their morphology were carried out essentially as described previously (Lai et al., 2014). Sequencing of 16S rRNA, *gyrB*, *rpoB* and *pycA* genes was carried out as described by Liu et al. (2013). Biochemical tests detecting carbon source utilization, enzymic activities and antibiotic resistance of these strains were performed using the VITEK 2 microbial identification system (bioMérieux) in accordance with the manufacturer’s instructions. Pulsed-field gel electrophoresis (PFGE) analysis was conducted as described by Dobritsa et al. (2010).

Sequencing of the *B. aerius* MTCC 7303<sup>T</sup> 16S rRNA gene showed that the strain may not represent *B. aerius*. The 16S rRNA gene sequence was identical to that of *B. altitudinis* DSM 21631<sup>T</sup> (accession no. AJ831843.1), but its similarity to the sequence AJ831843.1 used during description of *B. aerius* was not more than 95.7%. Strain MTCC 7303<sup>T</sup> also shared 100% similarities of the *gyrB*, *rpoB* and *pycA* gene sequences with those of *B. altitudinis* DSM 21631<sup>T</sup>. This level of similarity of housekeeping genes is certainly higher than the interspecies similarity gap of 95–96% demonstrated by comparing concatenated sequences of *gyrB*, *rpoB* and *pycA* genes of species from the *B. pumilus* group (Liu et al., 2013). Therefore, strain MTCC 7303<sup>T</sup> should be affiliated with *B. altitudinis*.

The phylogenetic placement of the strain is supported by the results of biochemical tests of strains MTCC 7303<sup>T</sup> and *B. altitudinis* DSM 21631<sup>T</sup>. There were no differences between the two strains when VITEK 2 BCL card test reactions used for identification of species of the genus *Bacillus* and members of related genera were compared. Increasing the total number of different tests to 115 using GP (Gram-positive), GN (Gram-negative) and YST (yeasts) cards did not give any differentiating reaction either.

Moreover, according to the results of PFGE analysis, strain MTCC 7303<sup>T</sup>, which was designated to represent the type strain of *B. aerius*, appeared to be a clone of the type strain of *B. altitudinis*. The PFGE patterns of DNA fragments generated by digesting the genomic DNAs of strains MTCC 7303<sup>T</sup> and *B. altitudinis* DSM 21631<sup>T</sup> with four restriction endonucleases, *ApaI*, *AscI*, *SmaI* and *NotI* were indistinguishable (Fig. 1).

Summarizing the data of the polyphasic characterization of strain MTCC 7303<sup>T</sup>, which is assumed to be the type strain of the species *B. aerius*, and the results of our search for other strains of this species, we may only conclude that this strain does not conform to the species description and to the best of our knowledge other strains which might represent the type strain of *B. aerius* have been lost as well. Therefore, in accordance with Rule 18c of the International Code of Nomenclature of Bacteria (Lapage et al., 1992), we propose that the Judicial Commission places the name *Bacillus aerius* on the list of rejected names if a suitable replacement type strain or a neotype cannot be found or proposed, respectively, within two years of the publication of this Request for an Opinion.

During preparation of the manuscript of this article, a Request for an Opinion was published online (Branquinho et al., 2015) in which the authors, based on unsuccessfully trying to receive *B. aerophilus* and *B. stratosphericus* type strains from the researchers describing these species and collections where the strains were deposited, proposed

Fig. 1. PFGE patterns of strains MTCC 7303<sup>T</sup>, MTCC 7304<sup>T</sup> and *B. altitudinis* DSM 21631<sup>T</sup> generated after digestion of their DNAs with restriction enzymes *ApaI*, *SmaI*, *NotI* and *AscI*. 
that the Judicial Commission places the names *Bacillus aerophilus* and *Bacillus stratosphericus* on the list of rejected names. Our results of polyphasic analysis of strains MTCC 7304\textsuperscript{T} and MTCC 7305\textsuperscript{T}, designated to represent the type strains of *B. aerophilus* and *B. stratosphericus*, respectively, and still available, at least for Indian scientists, support this Request for an Opinion. Like strain MTCC 7303\textsuperscript{T}, strain MTCC 7304\textsuperscript{T} has 16S rRNA, gyrB, rpoB and pycA gene sequences which are identical to those of *B. altitudinis* DSM 21631\textsuperscript{T}, and its biochemical traits and PFGE patterns (see Fig. 1) are indistinguishable from those of strain MTCC 7303\textsuperscript{T} and *B. altitudinis* DSM 21631\textsuperscript{T}. Therefore, *B. aerophilus* MTCC 7304\textsuperscript{T} is indeed another clone of the type strain of *B. altitudinis*. Cells of the bacterium supplied by MTCC as *B. stratosphericus* MTCC 7305\textsuperscript{T} were Gram-negative, motile rods and the strain was identified based on the results of 16S rRNA and rpoB genes sequencing as phylogenetically most closely related to *Proteus vulgaris* and *Proteus penneri*.

We would also like to present to the attention of the Judicial Commission and/or the *International Journal of Systematic and Evolutionary Microbiology* (IJSSEM) editors some other issues which we encountered during this study of the status of the species *B. aerius*, *B. aerophilus* and *B. stratosphericus* that need to be corrected. Firstly, the refusal by the MTCC to provide strains to other countries, which were submitted to the collection with the aim of receiving a certificate of deposit by a depositor for the subsequent publication of the description of a novel taxon, is in contradiction with Rule 30(3a) of the Code requiring availability of type materials in public collections. The importance of the rule was emphasized once again in the current recommendations on description of prokaryote taxa (Tindall *et al.*, 2010). The MTCC is clearly familiar with these requirements; a statement in its certificates of deposit claim, ‘This strain is available in the publicly accessible section of the MTCC and restrictions have not been placed on access to information concerning the presence of this strain in the MTCC’, but, nevertheless, the MTCC declined our requests for the strains. It is understandable that IJSSEM editors do not have authority to order the MTCC or other collections to submit strains abroad, but, at least, IJSSEM could prevent such situations by rejecting acceptance as valid of certificates of deposit from collections which do not follow the rule.

Secondly, unlike the MTCC, the JCM, the second collection where bacterial cultures were deposited as the type strains of *B. aerius*, *B. aerophilus* and *B. stratosphericus* before the species description was published did not list them in their catalogue, but the reason for their absence was not indicated. The collection did not respond to several requests for the strains five years ago and only recently informed us that the strains were characterized by the JCM and it was found that the strains were incorrect. We consider that it would be more constructive, both for collections and their customers, that when the collections responsible for storage of the strains submitted in accordance with Rule 30(3a) establish that the deposited strains are not viable or are not considered to be the original strains, they should put the corresponding notes in catalogues and inform the LPSN and StrainInfo (http://www.straininfo.net) websites. This would prevent a waste of time for scientists, and making reasons for the loss of a type strain publicly accessible might help the collection to recover the strain.

Thirdly, sequences in GenBank may be corrected by a depositor by submitting new versions. Unfortunately, use of this option by S. Shivaji resulted in new versions of *B. aerophilus* and *B. stratosphericus* 16S rRNA gene sequences changed to such a level that they may only represent different species. However, because, in general, new versions are supposed to be more accurate than previous ones, sequences AJ831844.2 and AJ831841.2 continued to be considered as representing *B. aerophilus* and *B. stratosphericus*. There are now not less than 145 and 200 16S rRNA gene sequences in GenBank from bacterial strains which were identified as *B. aerophilus* and *B. stratosphericus*, respectively, based on their similarity with the new sequence versions. Even NCBI staff in collaboration with an International Consortium of rRNA Databases accepted sequences AJ831844.2 and AJ831841.2 as the reference 16S rRNA gene sequences (NR_042339 and NR_042336, respectively) for *B. aerophilus* and *B. stratosphericus*. To avoid such mistakes, it would be desirable to limit the possibility of radical changes of sequences which were used as part of a novel species description, without the relevant peer-reviewed publications.

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**References**


