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Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): Proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov. as *Lelliottia nimipressuralis* comb. nov. and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*☆

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ABSTRACT

The taxonomy of *Enterobacter* has a complicated history, with several species transferred to and from this genus. Classification of strains is difficult owing to its polyphyletic nature, based on 16S rRNA gene sequences. It has been previously acknowledged that *Enterobacter* contains species which should be transferred to other genera. In an attempt to resolve the taxonomy of *Enterobacter*, MLSA based on partial sequencing of protein-encoding genes (*gyrB*, *rpoB*, *infB* and *atpD*) was performed on the type strains and reference strains of *Enterobacter*, *Cronobacter* and *Serratia* species, as well as members of the closely related genera *Citrobacter*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Mangrovibacter*, *Raoultella* and *Yokenella*. Phylogenetic analyses of the concatenated nucleotide sequences revealed that *Enterobacter* can be divided into five strongly supported MLSA groups, suggesting that the species should be reclassified into five different genera. Further support for this was provided by a concatenated amino acid tree, phenotypic characteristics and fatty acid profiles, enabling differentiation of the MLSA groups. Three novel genera are proposed: *Lelliottia* gen. nov., *Pluralibacter* gen. nov. and *Kosakonia* gen. nov. and the following new combinations: *Lelliottia nimipressuralis* comb. nov., *Lelliottia amnigena* comb. nov., *Pluralibacter gergoviae* comb. nov., *Pluralibacter pyrinus* comb. nov., *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov., *Kosakonia arachidis* comb. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov. Additionally, the novel epithet *Cronobacter zurichensis* nom. nov. is proposed for the reclassification of *Enterobacter turicensis* into the genus *Cronobacter*, as *Cronobacter turicensis* (Iversen et al., 2008) is already in use.

☆ *Note:* The GenBank/EMBL accession numbers for sequences generated in this study are as follows: JX424847–JX424976, JX494747–JX494748 (*atpD*), JX424977–JX425105, JX494749–JX494750 (*gyrB*), JX425106–JX425235, JX494751–JX494752 (*infB*) and JX425236–JX425361, JX494753–JX494754 (*rpoB*).

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Introduction

The genus *Enterobacter* was created in 1960 to resolve the classification of strains previously identified as "*Aerobacter aerogenes*" and "*Aerobacter cloacae*", also known as "Cloaca B" and "Cloaca A" respectively [24]. However, strains belonging to *Enterobacter* were being isolated as early as 1885 as "*Bacillus lactis aerogenes*". A group of strains known as "*Aerobacter liquefaciens*" was included in *Enterobacter* by Ewing and Fife [13] but also concurrently transferred to the genus *Serratia* as *S. liquefaciens* by Bascomb et al. [1]. The taxonomy of *Enterobacter* has a long and confusing history, with several transfers of species over the past 20 years. In the late 1980s, *Erwinia cancerogenus*, *Erwinia nimipressuralis* and *Erwinia dissolvens* were transferred to *Enterobacter* as new combinations [7,11]. In 1989, *Enterobacter agglomerans* was transferred from *Enterobacter* to the novel genus *Pantoea* [15] and *Enterobacter taylorae* was found to be a heterotypic synonym of *Enterobacter cancerogenus* [17,46]. Later, *Enterobacter dissolvens* was reassigned to *Enterobacter cloacae* as *E. cloacae* ssp. *dissolvens* [22] and *Enterobacter intermedius* was transferred to the genus *Kluyvera* as *K. intermedia* [41]. *Enterobacter aerogenes* and *Klebsiella mobilis* are considered homotypic synonyms, and *E. aerogenes* is even treated as a member of the genus *Klebsiella* in Bergey's Manual of Systematic Bacteriology [19] as it is more related to *K. pneumoniae* than *E. cloacae*. However, *E. aerogenes* is still regarded as a member of *Enterobacter* in many taxonomic studies, which should be discouraged to prevent further confusion in the taxonomy of this genus. More recently *Enterobacter sakazakii* was transferred to a novel genus, *Cronobacter*, with several novel species that were delineated from previously described biogroups [27]. In addition to these rearrangements, several *Enterobacter* species including *E. cloacae*, *E. asburiae*, *E. hormaechei*, *E. kobei* and *E. nimipressuralis* are presumed to belong to what is known as the "*E. cloacae* complex" based on genotypic and phenotypic relatedness [22]. The majority of species belonging to the "*E. cloacae* complex" are of clinical relevance, compared to the remaining species which are associated with plants, foods and environmental sources.

Currently, 19 species (excluding *E. aerogenes*) are recognized in the genus *Enterobacter* making it one of the largest genera within the family *Enterobacteriaceae*. *Enterobacter* is also one of the most rapidly expanding genera within this family, with 50% of the novel species descriptions taking place in the last decade. The older species were assigned to the genus based on DNA-DNA hybridization values and phenotypic data, whereas the more recently described taxa rely on 16S rRNA gene- and *rpoB*-sequencing for genus allocation.

Like many genera within the *Enterobacteriaceae*, *Enterobacter* has been shown to be polyphyletic based on the 16S rRNA gene [35,49], making it difficult to assign novel species to *Enterobacter* unless the strains cluster with the type species (*E. cloacae*) of the genus. Recently more researchers are relying on *rpoB* sequence analysis for classification of *Enterobacter* isolates [35,50,56] as it provides a higher phylogenetic resolution compared to that of the 16S rRNA gene. However, even the increased resolution of the *rpoB* gene fails to resolve *Enterobacter* and its closest phylogenetic relatives in monophyletic clades.

Moreover, even a multilocus sequence analysis (MLSA) scheme recently developed for *Cronobacter* and related taxa demonstrated the polyphyletic nature of *Enterobacter* when sequences of the protein-encoding genes *recN*, *rpoA* and *thdF* were analyzed [34]. If the major aim of taxonomy is to circumscribe taxa in monophyletic groups [44], then it is highly likely that *Enterobacter* currently contains species which should be reassigned to existing or novel genera. This view has been previously expressed [29], but data to support such a major taxonomic re-arrangement has been insufficient.

MLSA, based on partial sequencing of the protein-encoding genes *gyrB*, *rpoB*, *infB* and *atpD*, has been used to address several taxonomic issues in the genera *Pantoea*, *Tatumella* [3,4], *Dickeya* and *Brenneria* [5]. Phylogenetic analyses using these four genes delineated species into well-defined clades and were the basis for the transfer of several species to existing or novel genera. In the present study this MLSA scheme was extended to include reference strains belonging to the validly published species of *Enterobacter*, *Cronobacter* and *Serratia*, as well as selected species of the closely related genera *Citrobacter*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Mangrovibacter*, *Raoultella* and *Yokenella*. The *Enterobacter* reference strains were examined using a polyphasic approach including MLSA, DNA–DNA hybridizations, phenotypic tests and fatty acid analyses in a taxonomic re-evaluation of the genus *Enterobacter*.

Materials and methods

Strains investigated and DNA extraction

Between two and four strains (including the type strain) were selected from different locations and sources for each *Enterobacter*, *Cronobacter* and *Serratia* species. Originally, four to five strains per species were to be included in the present study. However, several *Enterobacter* and *Serratia* species are described based on a single strain, whereas for other species there is a lack of well-characterized strains publicly available. In addition, on more than one occasion strains were found to be misidentified. A detailed summary of the strains used in this study is listed in Suppl. Table S1. The majority of strains were provided by the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm). Additional strains were obtained by BCCM/LMG from CCUG, CCM, DSMZ, ICMP, KCTC and NCPPB. Genomic DNA for sequencing was extracted using an alkali extraction method as previously described [39].

Multilocus sequence analysis (MLSA)

Amplification and partial sequencing of the *gyrB*, *rpoB*, *infB* and *atpD* genes was performed on all strains using the primers and conditions as described previously [2]. A lower annealing temperature of 46 °C was found to improve amplification of the *gyrB* gene. PCR- and sequencing-setup, and purification steps were carried out using a Genesis Workstation 200 platform (Tecan). The resulting nucleotide sequence lengths were: *gyrB* = 742 bp, *rpoB* = 637 bp, *infB* = 615 bp and *atpD* = 642 bp.

Phylogenetic analysis

The best-fit evolutionary model for each individual gene data set, as well as the concatenated nucleotide sequence data set of all four protein-encoding genes, were determined in Modeltest 3.7 [43] and Mr Modeltest 2.3 [40]. The resulting parameters were used to construct maximum likelihood, neighbour joining and Bayesian phylogenies in Phyml 3.0 [20], Paup 4.0b10 [51], and Mr Bayes 3.2 [25], respectively. Bootstrap support with 1000 replicates and Bayesian posterior probability with 2 million generations, were generated to estimate the reliability of the clusters. As a high congruence in topology was observed between the three trees, only maximum likelihood trees are shown. The alignments of all four protein-encoding genes were examined for signature nucleotides that can distinguish between the different taxa under consideration. The confidence of alternative tree topologies based on the single gene datasets, as well as the concatenated dataset, was evaluated by the Approximately Unbiased (AU) test, the Kishino–Hasegawa (KH) test and the Shimodaira–Hasegawa (SH) test in Consel [47].