Description of Siccibacter colletis sp. nov., a novel species isolated from plant material, and emended description of Siccibacter turicensis

Emily E. Jackson,1 Naqash Masood,1 Khaled Ibrahim,1 Noémie Urvoy,2 Sumyya Hariri1 and Stephen J. Forsythe1

1School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK
2Université Paris Est Créteil, Département Génie Biologique, 61 Avenue du Général de Gaulle, 94000 Créteil, France

A re-evaluation of the taxonomic position of two strains, 1383T and 2249, isolated from poppy seeds and tea leaves, which had been identified as Siccibacter turicensis (formerly Cronobacter zurichensis), was carried out. The analysis included phenotypic characterization, 16S rRNA gene sequencing, multilocus sequence analysis (MLSA) of five housekeeping genes (atpD, fusA, glnS, gyrB and infB; 2034 bp) and ribosomal MLSA (53 loci; 22 511 bp). 16S rRNA gene sequence analysis and MLSA showed that the strains formed an independent phylogenetic lineage, with Siccibacter turicensis LMG 23730T as the closest neighbour. Average nucleotide identity analysis and phenotypic analysis confirmed that these strains represent a novel species, for which the name Siccibacter colletis sp. nov. is proposed. The type strain is 1383T (=NCTC 14934T=CECT 8567T=LMG 28204T). An emended description of Siccibacter turicensis is also provided.

The genera Siccibacter, Cronobacter and Franconibacter are members of the family Enterobacteriaceae of the class Gammaproteobacteria and are composed of various former species of the genus Enterobacter. Although these three genera are closely related, only species of Cronobacter have been linked to cases of human illness (FAO/WHO, 2008). Members of the genus Siccibacter have been described as facultatively anaerobic, Gram-negative, weakly oxidase-positive, catalase-positive, non-spore-forming rods that are motile, do not produce acetoin (Voges–Proskauer test) and are positive for the methyl red test (Stephan et al., 2014).

Confusions resulting from phenotypic and biochemical identification of members of the family Enterobacteriaceae have been described. For example, a number of strains of Enterobacter cloacae and Enterobacter hormaechei isolated from human infections were mistakenly assigned to the genus Cronobacter using phenotyping tests (Caubilla-Barron et al., 2007; Townsend et al., 2008). Consequently, it is difficult to describe novel members of this family solely on the basis of biochemical traits described previously in the literature. Phenotyping tests were not the primary means of species description in the recent naming of Cronobacter condimenti, Cronobacter helveticus, Cronobacter pulveris or Cronobacter zurichensis (Joseph et al., 2012b; Brady et al., 2013). Instead, multilocus sequence analysis (MLSA) and 16S rRNA gene sequencing were used to differentiate the species; however, 16S rRNA gene sequencing has limited application to the genus Cronobacter because of the high interspecies similarity, ranging from 97.8 to 99.7 %, and microheterogeneities in the gene (Iversen et al., 2008; Joseph et al., 2012a). Additionally, 16S rRNA gene sequencing showed high levels of similarity between Cronobacter sakazakii and Citrobacter koseri (97.8 %) and Cronobacter sakazakii and Enterobacter cloacae (97.0 %; Iversen et al., 2004). Therefore, fusA sequence analysis is commonly used for identification of species of the genus Cronobacter, the phylogeny of which reflects the whole-genome phylogeny of the genus Cronobacter (Joseph et al., 2012a, c). MLSA and fusA sequence analyses can also be applied to the genus Siccibacter, as a result of its close relationship to and previous inclusion in the genus Cronobacter.

MLSA based on housekeeping genes, including fusA, has proven to be a useful tool for taxonomic analysis of the

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**Abbreviations:** ANI, average nucleotide; MLSA, multilocus sequence analysis; MLST, multilocus sequence typing.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA, atpD, fusA, glnS, gmrB, gyrB, infB and ppsA gene sequences of strain 1383T are LK054215–LK054222, respectively. These sequences can also be accessed from the PubMLST Cronobacter multilocus sequence typing database (http://pubmlst.org/cronobacter). The accession number for the genome sequence of strain 1383T is JMS000000000.

Two supplementary tables and a supplementary figure are available with the online Supplementary Material.

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**Correspondence**

Stephen J. Forsythe
stephen.forsythe@ntu.ac.uk

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Enterobacteriaceae and was found to be more effective than phenotyping for speciation of members of the genus Cronobacter (Jolley et al., 2004; Jolley & Maiden, 2010; Joseph & Forsythe, 2012). Joseph et al. (2012b) used a seven-locus MLSA (atpD, fusA, glnS, gltB, gyrB, infB and ppsA; 3036 bp concatenated sequence length) for the definition of two novel species of the genus Cronobacter: Cronobacter universalis and Cronobacter condimenti. In contrast, Brady et al. (2013) only used four loci (atpD, gyrB, infB and rpoB) to support their reclassification of Enterobacter helveticus, Enterobacter pulveris and Enterobacter turicensis as Cronobacter helveticus, Cronobacter pulveris and Cronobacter zurichensis, respectively. This reclassification is disputed by Stephan et al. (2014), who proposed two new genera containing the species Fracionibacter helveticus, Fracionibacter pulveris and Siccibacter turicensis for the same former species of the genus Enterobacter, based on single-nucleotide polymorphism analysis of whole-genome sequences.

The present investigation determined the taxonomic position of two strains (1383T and 2249) that had previously been assigned to the species Cronobacter zurichensis and Siccibacter turicensis (Brady et al., 2013; Stephan et al., 2014). Both strains were isolated while screening food products for the presence of members of the genus Cronobacter. Strain 1383T was isolated in 2011 from poppy seeds and strain 2249 was isolated from pear and vanilla herbal tea bags, both purchased in the UK (Jackson et al., 2014). The strains were isolated at 37 °C using Enterobacteriaceae enrichment broth (CM1115; Oxoid Thermoscientific) and then plated onto Draggan–Forsythe–Iversen Enterobacter sakazakii chromogenic agar (CM1055; Oxoid Thermoscientific). Typical blue–green colonies, indicating z-glucosidase activity, were selected for identification. Following the taxonomic revisions of Enterobacter turicensis, both strains were classified as Cronobacter zurichensis (Brady et al., 2013) and then Siccibacter turicensis (Stephan et al., 2014). This study re-evaluated this identification using a wider range of physiological, phenotyping and DNA-based techniques, including whole-genome sequence analysis.

Phenotypic analysis using API 20 E (bioMérieux) provisionally identified strain 1383T as Klebsiella pneumoniae subsp. ozaenae. ID 32E (bioMérieux) provisionally identified the strain as Enterobacter sakazakii, prior to the taxonomic recognition of the genus Cronobacter (Iversen et al., 2007). However, 16S rRNA gene sequence comparisons showed that the nearest match was to Enterobacter turicensis, which was later reclassified as Cronobacter zurichensis, then Siccibacter turicensis (Brady et al., 2013; Stephan et al., 2007, 2014).

Phenotypic analysis using API 20 E (bioMérieux) provisionally identified strain 2249 as a strain of Erwinia, whereas ID 32E (bioMérieux) provisionally identified the strain as Escherichia vulneris. However, fusA gene sequence analysis (438 bp) showed the nearest match was to Siccibacter turicensis strain 1383 in the Cronobacter PubMLST database, with 1 nucleotide difference at position 270, and 22 differences from the fusA sequence of Siccibacter turicensis LMG 23730T.

The original description of Siccibacter turicensis (then called Enterobacter turicensis) utilized Biotype 100 test strips, which are no longer available (Stephan et al., 2007). Therefore, the phenotypic tests used to re-evaluate strains 1383T and 2249 were selected from Iversen et al. (2006a, b, 2007, 2008), Stephan et al. (2007, 2014), Joseph et al. (2012b) and Brady et al. (2013). These tests included catalase and oxidase activity, nitrate reduction, acid production from sugars, malonate and salic acid utilization, production of indole from tryptophan, motility, gas from D-glucose, Voges–Proskauer test, z-glucosidase, pigment production on tryptone soy agar (TSA) (CM0131; Oxoid Thermoscientific) at 25 °C and colony morphology on MacConkey agar. Acid production from carbohydrates was determined in nutrient broth supplemented with phenol red and the following substrates (1%, w/v): myo-inositol, putrescine, lactulose, 4-aminoxybutyrate, maltitol and trans-aconitate. Sialic acid utilization, motility, gas from glucose and growth on TSA and MacConkey agar were assessed using conventional methods. The remaining tests (production of indole and hydrogen sulfide, Voges–Proskauer test, z-glucosidase, β-galactosidase, ornithine decarboxylase, hydrolysis of gelatin and urea and acid production from D-mannitol, D-sorbitol, L-ramnose, inositol, sucrose and L-arabinose) were performed in parallel using the API 20 E and ID 32 E systems (bioMérieux). Fermentation/oxidation of 49 carbohydrates was tested using the API 50 CH system (bioMérieux), according to the manufacturer’s instructions. The activities of various enzymes were determined by using the API ZYM system (bioMérieux). Appropriate positive and negative controls were included. All tests were performed at 37 °C and incubated for 24 h. Motility was assessed in motility medium (10 g tryptose, 5 g NaCl and 5 g agar 1−1, pH 7.2±0.2) (Iversen et al., 2007). Type strains of all species of the genera Siccibacter, Fracionibacter and Cronobacter were evaluated under identical conditions for the selected differential tests included in Table 1.

Strains 1383T and 2249 were found to differ from strains of their closest relative, Siccibacter turicensis, in gas production from D-glucose, in the utilization of sialic acid and in two enzyme activities, acid phosphatase and N-acetyl-β-glucosaminidase (Table 1).

The susceptibility of strains 1383T and 2249 to 12 antibiotics was assessed according to the standards and procedures of the British Society for Antimicrobial Chemotherapy (BSAC, 2014), using Escherichia coli NCTC 10418 as the control organism. The strains were classified as susceptible, intermediate or resistant according to BSAC criteria. The following antibiotic-containing discs obtained from Mast Diagnostics were tested: amikacin (AK30), ampicillin (AP10), amoxicillin plus clavulanic acid (AUC30), cefotaxime (CTX30), cefuroxime (CMX30), cefazidime (CAZ30), chloramphenicol (C30), ciprofloxacin (CIP1), doxycycline

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MLSA of five housekeeping genes, strains 1383T and 2249

Taxa: 1, Siccibacter colletis sp. nov. strains 1383T and 2249; 2, Siccibacter turicensis (n=2); 3, Franconibacter helveticus (n=2); 4, Franconibacter pulvis (n=6); 5, Cronobacter sakazakii (n=163); 6, Cronobacter malonaticus (n=22); 7, Cronobacter turicensis (n=8); 8, Cronobacter universalis (n=4); 9, Cronobacter murytjensi (n=7); 10, Cronobacter dublinensis (n=8); 11, Cronobacter condimenti 1330T. Data for reference taxa were from Iversen et al. (2007, 2008), Stephan et al. (2007, 2008, 2014), Joseph et al. (2012b), Brady et al. (2013) and the current study. +, Positive; V, variable (25–75 % positive); –, negative; ND, no data available. Key traits for differentiation of species of the genus Siccibacter are shown in bold. Reactions of type strains are shown in parentheses. All strains are positive for acid production from D-glucose and negative for H2S production.

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(DXT30), gentamicin (GM10), imipenem (IM10) and trimethoprim plus sulfamethoxazole (TS25). Both strains 1383T and 2249 were found to be resistant to doxycycline. Additionally, strain 1383T was resistant to ciprofloxacin and showed intermediate resistance to cefotaxime. Both strains were sensitive to all other antibiotics tested.

It is recognized that the former reliance on phenotyping to define members of the genus Cronobacter and closely related species and incorrect speciation of biotype strains has led to contradictions in the biochemical descriptions of species of the genus Cronobacter (Baldwin et al., 2009; Joseph et al., 2013). Hence, the DNA-sequence-based techniques multilocus sequence typing (MLST), ribosomal MLST (rMLST), average nucleotide identity (ANI) and whole-genome analysis were used in this study as more reliable means of defining the novel species of the genus Siccibacter.

For phylogenetic studies of the 16S rRNA gene and for MLSA of five housekeeping genes, strains 1383T and 2249 were cultured on TSA (CM0131; Oxoid Thermoscience) at 37 °C. DNA was extracted from a single colony by using a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) following the manufacturer’s instructions. Primers and conditions for amplification and sequencing of the 16S rRNA (1361 bp), ATPD (390 bp), fusA (438 bp), glnS (363 bp), gyrB (402 bp) and infB (441 bp) genes have been described previously (Iversen et al., 2007; Baldwin et al., 2009). Concatenated sequences (ATPD, fusA, glnS, gyrB and infB; 2034 bp) were aligned in MEGA software version 5.2 (Tamura et al., 2011) using the CLUSTAL W algorithm. Genetic distances and clustering were determined using Kimura’s two-parameter model (Kimura, 1980) and evolutionary trees were reconstructed by the neighbour-joining method (Saitou & Nei, 1987). Trees were also reconstructed using the maximum-likelihood method (Tamura et al., 2011) to ensure the robustness of the analysis. Stability of relationships was assessed by the bootstrap method (1000 replicates). The phylogenetic tree of the full 16S rRNA gene sequence (1451 bp; Fig. S1), available in the online Supplementary Material) was reconstructed using new and previously available 16S rRNA gene sequences (trimmed length 1361 bp) available in GenBank for all species of the genera Siccibacter, Cronobacter and Franconibacter. Phylogenetic trees for the five-locus MLSA (Fig. 1) used the existing curated sequences at the Cronobacter PubMLST database (http://pubmlst.org/cronobacter) initially created by Baldwin et al. (2009) and the new sequences obtained in this study.

The 16S rRNA gene tree (Fig. S1) showed that strain 1383T clustered with Siccibacter turicensis DSM 23730T and formed a cluster separate from the genera Cronobacter and Franconibacter. This supports the proposal of Stephan
et al. (2014) that the species *Cronobacter zurichensis* (former *Enterobacter turicensis*) should be recognized as a member of a new genus; the genus *Siccibacter*, containing one species, *Siccibacter turicensis*. Additionally, *Siccibacter turicensis* and the novel species represented by strain 1383T appear on two separate branches of the cluster, and strong bootstrap support (81%) suggests that they should be classified as two separate species. The *Siccibacter* cluster was close to *Enterobacter cloacae* and the genera *Citrobacter* and *Kosakonia*. *Franconibacter helveticus* and *Franconibacter pulveris* clustered with the genus *Cronobacter*. Thus, based on the 16S rRNA gene sequence analysis, it is unclear whether the latter two species (formerly *Enterobacter helveticus* and *Enterobacter pulveris*, respectively) belong to the genus *Cronobacter* or not (Brady et al., 2013; Stephan et al., 2014). It should be noted that 16S rRNA gene sequencing has been shown to be unreliable for species differentiation of *Cronobacter*; therefore, the five-locus MLSA was considered to be more reliable (Jolley et al., 2004; Jolley & Maiden, 2010; Joseph & Forsythe, 2012; Joseph et al., 2012a).

This apparent contradiction was further investigated using MLSA. The five-locus MLSA tree showed that the genera *Siccibacter*, *Franconibacter*, and *Cronobacter* formed discrete clusters separate from the genera *Citrobacter* and *Enterobacter* (Fig. 1). Strains 1383T and 2249 clustered near *Siccibacter turicensis* LMG 23730T, but on their own branch with strong bootstrap support (100%), again suggesting that strains 1383T and 2249 belong to a novel

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**Fig. 1.** Neighbour-joining phylogenetic tree based on concatenated *atpD*, *fusA*, *glnS*, *gyrB* and *infB* sequences (2034 bp), showing the position of strains 1383T and 2249 within the genera *Siccibacter*, *Franconibacter*, *Cronobacter*, *Citrobacter* and *Enterobacter*. Sequences were accessed via the database at http://pubmlst.org/cronobacter. Bootstrap percentages based on 1000 replications are shown at branch nodes. Bar, 1 substitution per 100 nucleotide positions.
species and not to *Siccibacter turicensis*. This analysis also supported the recognition of the two genera *Siccibacter* and *Franconibacter*, as proposed by Stephan *et al.* (2014), and not the inclusion of the former species *Enterobacter helveticus*, *Enterobacter turicensis* and *Enterobacter pulveris* within the genus *Cronobacter*, as proposed by Brady *et al.* (2013).

Analysis of the sequences of genes encoding ribosomal proteins (rMLST) has been proposed as a means of integrating microbial genealogy and typing (Jolley *et al.*, 2012). For rMLST analysis, 32 whole-genome sequences from members of the genera *Siccibacter*, *Franconibacter* and *Franconibacter* were analysed using the Analysis/Genome Comparator option with default settings in the *Cronobacter* PubMLST database (http://pubmlst.org/cronobacter). This tool extracts the ribosomal gene sequences from the selected genomes, and these sequences can then be used for phylogenetic analysis. The total concatenated length of the 53 loci was 22 511 bp.

Phylogenetic analysis of the rMLST sequences showed that strain 1383\(^T\) clustered with *Siccibacter turicensis* LMG 23730\(^T\), but on a unique branch of the tree (Fig. S2). As with the other phylogenetic analyses, strong bootstrap support (100 %) indicates that strain 1383\(^T\) does not belong to the species *Siccibacter turicensis* and should be assigned to a distinct species. Additionally, the genera *Siccibacter*, *Franconibacter* and *Cronobacter* formed discrete clusters that were separate from the genera *Citrobacter* and *Enterobacter* (Fig. S2). This analysis also supported the recognition of *Siccibacter* and *Franconibacter* as discrete genera separate from *Cronobacter* (Stephan *et al.*, 2014), as also shown using the five-locus MLSA.

ANI analysis compares whole-genome sequences *in silico* and has been proposed as a replacement for DNA–DNA hybridization as a measure of the degree of relatedness between two different genomes (Konstantinidis & Tiedje, 2005; Chun & Rainey, 2014), the threshold for species differentiation being 95–96 % (Richter & Rossello-Mora, 2009). There is, however, no accepted ANI value for genus demarcation (Kim *et al.*, 2014; Qin *et al.*, 2014). The ANI values for 11 whole-genome sequences of strains of the genera *Siccibacter*, *Franconibacter* and *Franconibacter*, including *Siccibacter turicensis* LMG 23730\(^T\) and strain 1383\(^T\), were determined using a web-based service (http://enve-omics.ce.gatech.edu/ani/) and are given in Table S1. The ANI value between strain 1383\(^T\) and *Siccibacter turicensis* LMG 23730\(^T\) was 87.2 %, which is below the threshold for species demarcation. Although there is no accepted ANI demarcation for the genus boundary, it was notable that the ANI was 84–86 % between members of the genera *Siccibacter*, *Franconibacter* and *Cronobacter*, as shown in Table S1.

16S rRNA gene sequencing, MLSA, ANI analysis and phenotypic characterization clearly differentiated strains 1383\(^T\) and 2249 from existing species of the genera *Siccibacter*, *Cronobacter* and *Franconibacter* and indicated that these strains constitute an independent lineage within the genus *Siccibacter*. Therefore, the novel species *Siccibacter colletis* sp. nov. is proposed to accommodate these strains.

**Description of *Siccibacter colletis* sp. nov.**

*Siccibacter colletis* (col.le’tis. L. gen. n. colletis of a kind of vervain, referring to the isolation of the type strain).

Cells are straight, Gram-negative, non-spore-forming, motile rods, approximately 2 × 1 μm. Facultatively anaerobic. Colonies on TSA incubated at 37 °C for 24 h are 2–3 mm in diameter, opaque, circular and yellow (strain 2249) or cream-coloured (strain 1383\(^T\)). Colonies are pale yellow to yellow and glossy on TSA incubated at 25 °C. Grows on MacConkey agar, producing pink-purple colonies with large halos. In TSB, grows at 42 °C (optimum 37 °C), but not at 5 °C. Produces catalase, z-glucosidase and β-galactosidase, and is weakly positive for oxidase. Does not produce gas from D-glucose. Does not produce indole from tryptophan or hydrogen sulfide. Does not produce acetoin (Voges–Proskaucer negative). Does not hydrolyse gelatin or urea. Negative for lysine decarboxylase and ornithine decarboxylase. Positive for methyl red test. Reduces nitrate. Utilizes dulcitol, aesculin, melibiose and L-rhamnose. Does not utilize malonate, melezitose, turanose, inositol, lactulose, *trans*-aconitate, putrescine, 4-aminobutyrate, maltitol or sialic acid. Produces acid from glucose, dulcitol, L-arabinose, cellobiose, lactose, L- rhamnose, D-mannitol, N-acetylgulosamine, salicin and 2-ketogluconate, but not from inositol, melezitose, sucrose, D- or L-fucose, adonitol, turanose, D-sorbitol or 5-ketogluconate. Does not produce acid phosphatase. Produces N-acetyl-β-glucosaminidase. Capable of growth in modified lauryl sulfate broth containing 1 M NaCl at 37 °C, but not at 42 °C. Resistant to doxycycline. The API 20 E and ID 32 E profiles of the type strain are 3204153 and 04077563310, respectively.

The type strain is 1383\(^T\) (=NCTC 14934\(^T\)=CECT 8567\(^T\)=LMG 28204\(^T\)), isolated from poppy seeds. The whole genome sequence of strain 1383\(^T\) has been deposited in the *Cronobacter* PubMLST database and in GenBank under accession number JMSQ00000000 (Masood *et al.*, 2014).

**Emended description of *Siccibacter turicensis***

Stephan *et al.* 2014


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nov. as Pluralibacter gergoviae comb. nov. and Pluralibacter pyrus
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,
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Cronobacter genomospecies 1, and of three subspecies, Cronobacter
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