Assignment of *Brevibacterium stationis* (ZoBell and Upham 1944) Breed 1953 to the genus *Corynebacterium*, as *Corynebacterium stationis* comb. nov., and emended description of the genus *Corynebacterium* to include isolates that can alkalize citrate

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Brevibacterium stationis ATCC 14403^T, *Corynebacterium ammoniagenes* ATCC 6872 and two clinical isolates were found to form a single taxon group consistent with the genus *Corynebacterium*, designated here as *Corynebacterium stationis* comb. nov. The type strain of *Corynebacterium stationis* is ATCC 14403^T = CCUG 43497^T = CIP 104228^T = DSM 20302^T = NBRC 12144^T = JCM 11611^T = VKM B-1228^T. These strains can utilize citrate; therefore, inclusion of *C. stationis* requires that the description of the genus *Corynebacterium* be amended to include citrate-positive strains.

Brevibacterium stationis was first described in 1944 as ‘*Achromobacter stationis*’ (ZoBell & Upham, 1944) and was later assigned to the genus *Brevibacterium* (Breed, 1953). Placement of this species in the genus *Brevibacterium* has been questioned since at least 1972 (Yamada & Komagata, 1972b), and the species was described by Jones & Keddle (1986) as a *species incertis sedis*. Chemotaxonomic features of *B. stationis* ATCC 14403^T include the presence of meso-diaminopimelic acid, cell-wall sugars arabinose and galactose, the presence of corynomycolates, menaquinone types MK-8(H₂) and MK-9(H₂) as sole respiratory quinones and cellular fatty acids of the straight-chain and monounsaturated types, rather than the branched-chain types associated with *Brevibacterium*, features actually consistent with the genus *Corynebacterium* (Jones & Keddle, 1986). Similar phenetic and chemotaxonomic features had been reported for *Brevibacterium ammoniagenes* (Cooke & Keith, 1927), which prompted its reassignment to the genus *Corynebacterium* (Collins,

1987). Differentiation of *B. stationis* ATCC 14403^T from phenotypically similar taxa has historically been difficult. In 1983, using comprehensive probabilistic methods to analyse a wide variety of microbial characteristics, this strain was assigned to cluster E-II, along with *C. ammoniagenes* strains ATCC 6871^T (=DSM 20306^T), ATCC 15137 and IFO 12072 (=ATCC 6872) (Seiler, 1983). In 1993, *B. stationis* ATCC 14403^T was assigned using phenetic-grouping analytical methods to ‘*C. ammoniagenes* cluster 49’, which included various reference *C. ammoniagenes* strains, including ATCC 6871^T and ATCC 6872 (Kämpfer & Seiler, 1993). *C. ammoniagenes* strains ATCC 6871^T and ATCC 6872 had been compared with each other by DNA–DNA hybridization and found to be closely related but not identical, and so were thought possibly to be members of different taxon groups (Jones & Keddle, 1986). All established reference strains of *B. ammoniagenes* which were not glutamic acid producers were transferred to *C. ammoniagenes* (Collins, 1987), including *C. ammoniagenes* ATCC 6872, which has been the subject of intense study in recent years with respect to various important industrial applications, including nucleoside and vitamin B₁₂ production (Wang *et al.*, 2007a, b).

In this study, two clinically derived isolates are reported that were most like members of the genus *Corynebacterium* except that they alkalized citrate as a sole carbon source, a feature not described previously for any *Corynebacterium*

Abbreviation: CFA, cellular fatty acid.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are FJ172666 (strain NML 03-0173), FJ172667 (*B. stationis* ATCC 14403^T), FJ172668 (NML 94-0424) and FJ648509 (*C. ammoniagenes* ATCC 6872) (16S rRNA gene) and FJ172669 (NML 03-0173), FJ172670 (NML 94-0424), FJ172671 (*B. stationis* ATCC 14403^T), FJ648510 (*C. ammoniagenes* ATCC 6872) and FJ648511 (*C. ammoniagenes* ATCC 6871^T) (partial *rpoB* gene).

species. Using 16S rRNA and partial *rpoB* gene sequencing, the isolates were found to be most closely related to *B. stationis* ATCC 14403^T and otherwise were most closely related to *C. ammoniagenes*, among species in the genus *Corynebacterium*. Subsequently, biochemical, chemotaxonomic and genetic features of the clinical isolates as well as reference strains of *B. stationis* and *C. ammoniagenes* were studied to determine precise phylogenetic relationships and to address the long-standing recommendation that *B. stationis* should be transferred to *Corynebacterium*.

Isolates NML 94-0424 and NML 03-0173 (identifiers of the Canadian National Microbiology Laboratory, Winnipeg, MB, Canada) were obtained from blood cultures from a 62-year-old male with a chest infection and a 66-year-old female, but with no further clinical information being extant. They were originally identified as being most like *Corynebacterium* species but, by 16S rRNA gene sequencing, had <98% identity with the closest relative *C. ammoniagenes*, and both strains alkalized citrate. *B. stationis* ATCC 14403^T and *C. ammoniagenes* strains ATCC 6871^T and ATCC 6872 were acquired from the American Type Culture Collection (Manassas, VA, USA).

Clinical and reference strains were studied morphologically and biochemically tested using conventional (tube) sugars and substrates as outlined previously (Bernard *et al.*, 2002a). In addition, API Coryne, API ZYM, API 20E and API 50CH strips were used and *apiweb* (<https://apiweb.biomerieux.com>) was queried as described by the manufacturer (bioMérieux) to see what identification would be provided based on the code generated by the API Coryne strip. For this study, Simmons' citrate was used to determine alkalization of citrate (Weyant *et al.*, 1996), but API 20E strips were also tested for utilization of citrate, one using a light and one a heavy bacterial suspension, and incubated as described by the manufacturer.

Antimicrobial susceptibility testing was performed by microbroth dilution according to methods recommended by the Clinical and Laboratory Standards Institute and interpretative criteria for *Corynebacterium* species (CLSI, 2006) using cation-adjusted Mueller–Hinton broth with 2.5% lysed horse blood and Sensititre STP5F and GPN3F plates (Trek Diagnostic). Antimicrobial agents tested included ampicillin, cefepime, cefotaxime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, clindamycin, daptomycin, erythromycin, ertapenem, gatifloxacin, gentamicin, levofloxacin, linezolid, meropenem, moxifloxacin, penicillin, quinupristin/dalfopristin, rifampicin, telithromycin, tetracycline, tigecycline, trimethoprim/sulfamethoxazole and vancomycin.

Cellular fatty acid (CFA) composition analysis was done using the Sherlock system (MIDI) as outlined previously except that version 4.5 of the operating software was used (Bernard *et al.*, 1991). CFAs were studied further using library generation system software (MIDI). Metabolic products were determined as described previously (Bernard *et al.*, 2002b). Clinical isolate NML 94-0424 was

also analysed for types and quantities of respiratory quinones, extracted as described by Karr *et al.* (1982) and analysed using an HP 1100 HPLC with UV detector and an in-house calibration mixture containing saturated and unsaturated menaquinones MK-6 to MK-10. Otherwise, descriptions of quinones and G+C content from *B. stationis* and *C. ammoniagenes* reference strains were reported previously (Jones & Keddle, 1986; Collins, 1987).

16S rRNA gene sequencing was done as outlined previously (Bernard *et al.*, 2002b), yielding 1400–1500 bp, and partial sequencing (400–430 bp) of the *rpoB* gene was performed as described by Khamis *et al.* (2004). 16S rRNA gene sequences were derived from this study or from GenBank, including accession no. AJ620367 from *B. stationis* LMG 21670^T. A reference sequence for *C. ammoniagenes* derived from CIP 101283^T, GenBank accession no. X84440, was used for comparative analysis. Sequences were aligned using CLUSTAL W and the relationship among aligned sequences was inferred using 1000 replications and neighbour-joining analysis software found in MEGA 4 (Kumar *et al.*, 2008), with the scale shown representing percentage sequence divergence. Partial *rpoB* sequences were analysed using neighbour-joining software in MEGA 4, and a sequence from a fragment of similar size (~420 bp) from *Corynebacterium tuberculostearicum* CCUG 45418^T served as an outgroup.

Strains NML 94-0424 and NML 03-0173, *C. ammoniagenes* ATCC 6872 and *B. stationis* ATCC 14403^T, collectively referred to here as the *B. stationis*-like group, were found to produce colonies that were 1 mm in diameter, raised and whitish at 24 h, but yellowish or tan-pigmented after 48 h or more. All strains grew well within 24 h at 35 °C. All were observed to have small, ovoid or medium-length, Gram-stain-positive rod-shaped cells without spores that occurred singly, in pairs or in 'V' forms and exhibited club shapes. No haemolysis was observed on sheep-blood agar (SBA). No strain demonstrated lipophilia. *C. ammoniagenes* ATCC 6871^T produced grey-white colonies at 24 h or older and, by Gram stain, was similar to *B. stationis* ATCC 14403^T. Strains of both species grew well on SBA at 25 °C in air, at 35 °C in atmospheres of 5% CO₂ and air or microaerophilically and at 42 °C in air, but not at 50 or 60 °C or at 35 °C under strictly anaerobic conditions. All strains were catalase-positive and oxidase-negative. Biochemical results and enzymes detected are summarized in Table 1; most notably, all strains alkalized citrate within 24–48 h. Using the API 20E strip, citrate alkalization was positive when a heavy inoculum was used, equivalent to a 5 McFarland standard, but not when a light suspension was used. Citrate alkalization has not been described previously for members of *Corynebacterium sensu stricto* (Collins & Cummins, 1986) or for *B. stationis* or *C. ammoniagenes* (Jones & Keddle, 1986; Collins, 1987). Using the API Coryne system, *B. stationis* ATCC 14403^T and strains NML 94-0424 and NML 03-0173 generated a code of 3001304 and *C. ammoniagenes* strains ATCC 6871^T and ATCC 6872 generated a code of 1001304 (that is,

Table 1. Biochemical tests for *B. stationis*-like strains, *C. ammoniagenes* ATCC 6871^T and phenotypically similar species arising from use of *apiweb*, based on API Coryne codes

Taxa: 1, *B. stationis* ATCC 14403^T; 2, *C. ammoniagenes* ATCC 6872; 3, NML 94-0424; 4, NML 03-0173; 5, *C. ammoniagenes* ATCC 6871^T; 6, *C. renale* (usual source, bovines); 7, *C. pseudotuberculosis* (usual source, sheep). Data were obtained from this study and from Jones & Keddle (1986), Collins (1987), Collins & Cummins (1986) and Funke & Bernard (2007). ND, No data available; +, positive using tube sugars within 6 days (the same substrates were weakly reactive for *B. stationis* and *C. ammoniagenes* strains using API 50CH or API Coryne panels); –, negative/not reactive; d, variable depending on the strain. All taxa were positive for fermentation of glucose and fructose and urease activity. All strains were negative for fermentation of xylose, mannitol, lactose, maltose, galactose, glycerol, glycogen, raffinose, salicin, trehalose and aesculin, gas from nitrate, nitrite reduction, indole production, methyl red/Voges–Proskauer reaction, gelatin hydrolysis, CAMP and CAMP inhibition (reverse CAMP), DNase activity, hydrolysis of casein and starch and lysine, arginine and ornithine decarboxylases. Enzymes from the API Coryne gallery that were not detected included pyrrolidonyl arylamidase, β -glucuronidase, β -galactosidase, α -glucosidase and *N*-acetyl- β -glucosaminidase. Enzymes tested from the API ZYM panel that were found to be occasionally reactive were leucine arylamidase and esterase; all strains were non-reactive in the API ZYM panel for alkaline phosphatase, esterase lipase, lipase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase.

Strain	1	2	3	4	5	6	7
Pigmentation at 48 h*	Y	Y	Y	Y	GW	Y	Y
Fermentation of:							
Ribose	+	+	+	+	+	ND	ND
Mannose	+	+	+	+	–	+	+
Citrate alkalization	+	+	+	+	+	–	–
Nitrate reduction	+	+	+	+	+	–	d
Tyrosine hydrolysis	+	+	+	+	+	–	–
Pyrazinamidase	+	–	+	+	–	+	+
CAMP inhibition	–	–	–	–	–	–	+
Presence of diphtheria <i>tox</i> gene	–	–	–	–	–	–	+
Cysteine production	–	–	–	–	–	–	+

*GW, Grey-white; Y, yellowish.

pyrazinamidase was not detected), which, upon querying the *apiweb* database, related best to poor levels of confidence for the *Corynebacterium renale* group or *Corynebacterium pseudotuberculosis*, respectively. By either conventional tube substrates and/or using an API Coryne or API 50CH strip, both *C. ammoniagenes* ATCC 6871^T and the *B. stationis*-like strains were slowly or weakly reactive with glucose, fructose and ribose, reduced nitrate, produced urease and hydrolysed tyrosine. *B. stationis* ATCC 14403^T did not ferment CHO tube glucose, as

described previously (Yamada & Komagata, 1972a), but was reactive with glucose using the API Coryne and API 50CH panels. Historically, strains from both species have been found to hydrolyse hippurate (Jones & Keddle, 1986). Most strains were weakly reactive with fucose substrate found in the API 50CH strip, also described previously for *C. ammoniagenes* (Kämpfer & Seiler, 1993). The *B. stationis*-like strains, but not *C. ammoniagenes* ATCC 6871^T, fermented mannose weakly or slowly, unlike results found by ZoBell & Upham (1944) but as cited later by Jones & Keddle (1986). Previously described fermentation of sucrose, maltose and trehalose by *B. stationis*-like strains or *C. ammoniagenes* ATCC 6871^T (Yamada & Komagata, 1972a) was not observed here. Pyrazinamidase was reactive with three out of four *B. stationis*-like strains but was not detected for *C. ammoniagenes* ATCC 6871^T. Otherwise, these strains were non-reactive for detection of enzymes using the API ZYM panel, except for weak reactivity of *C. ammoniagenes* ATCC 6871^T and ATCC 6872 for leucine arylamidase and *B. stationis* ATCC 14403^T for esterase.

B. stationis ATCC 14403^T, strain NML 94-0424 and *C. ammoniagenes* ATCC 6872 were considered sensitive to all antimicrobials that have CLSI interpretative criteria. NML 03-0173 was observed to be resistant to erythromycin (MIC >4 $\mu\text{g ml}^{-1}$). *C. ammoniagenes* ATCC 6871^T was found to be sensitive to all antimicrobials except erythromycin (MIC >4 $\mu\text{g ml}^{-1}$) and clindamycin (MIC >2 $\mu\text{g ml}^{-1}$) (resistant), as well as having MICs of 2 $\mu\text{g ml}^{-1}$ (intermediate susceptibility) for cefotaxime and ceftriaxone.

CFAs were consistent with those determined previously for both *C. ammoniagenes* and *B. stationis* and with those of the genus *Corynebacterium* (Table 2) (Bernard *et al.*, 1991). Based on this analysis, CFAs were deemed to be quantitatively and qualitatively indistinguishable between *C. ammoniagenes* ATCC 6871^T and the *B. stationis*-like group. These CFAs were qualitatively unlike those described for members of *Brevibacterium sensu stricto*, where the majority of CFAs are of the branched-chain types (Jones & Keddle, 1986; Gruner *et al.*, 1994). Metabolic products found for *B. stationis*-like strains and *C. ammoniagenes* ATCC 6871^T were similar, with small volumes of lactic and succinic acids but not propionic acid being detected; the latter is only observed for about 25–30% of all *Corynebacterium* species (Bernard *et al.*, 2002a). Respiratory quinones for *B. stationis* ATCC 14403^T were described as comprising roughly equal amounts of MK-8(H₂) and MK-9(H₂) (Collins, 1987), and similar menaquinones were detected here for NML 94-0424 (data not shown), in contrast to members of *Brevibacterium sensu stricto*, which have MK-8(H₂) as the sole menaquinone (Jones & Keddle, 1986).

By 16S rRNA gene sequencing analysis, NML 94-0424, NML03-0173 and *C. ammoniagenes* ATCC 6872 had 99.2, 99.6 and 99.8% identity with *B. stationis* ATCC 14403^T, respectively; these four strains showed 98.1, 98.0, 98.0 and 97.9% 16S rRNA gene sequence identity, respectively, to

Table 2. CFA composition of strains of the *B. stationis*-like group and *C. ammoniagenes sensu stricto*

Values represent percentages of total fatty acids detected rounded to nearest integer; ND, not detected. TBSA, Tuberculostearic acid (10-methyl $C_{18:0}$). Strains of the *B. stationis*-like group also contained trace amounts of $C_{12:0}$ and all strains contained trace amounts of $C_{16:1\omega7c}$ (part of summed feature 3 in the MIDI system).

Fatty acid	<i>B. stationis</i> -like strains (n=4)	<i>C. ammoniagenes</i> ATCC 6871 ^T
$C_{14:0}$	2	1
$C_{16:1\omega9c}$	1	1
$C_{16:0}$	41	32
$C_{17:1\omega9c}$	4	9
$C_{17:1\omega8c}$	1	ND
$C_{17:0}$	2	4
$C_{18:1\omega9c}$	37	41
$C_{18:1\omega7c}$	2	ND
$C_{18:0}$	7	7
TBSA	2	2

C. ammoniagenes ATCC 6871^T (Fig. 1). Using criteria described by Stackebrandt & Ebers (2006), strains with 98.8 % identity or more can be considered members of the same taxon group. These relationships were corroborated by partial *rpoB* sequence analysis, where NML 94-0424, NML 03-0173 and *C. ammoniagenes* ATCC 6872 had 99.8, 98.6 and 99.5 % identity, respectively, to *B. stationis* ATCC 14403^T, but ~90 % identity to *C. ammoniagenes* ATCC 6871^T (Fig. 2). By partial *rpoB* gene sequence analysis, strains that demonstrate ≥95 % identity are considered members of the same taxon group (Khamis *et al.*, 2005).

Based on these studies, we conclude that *B. stationis* should be transferred to the genus *Corynebacterium*, as *Corynebacterium stationis* comb. nov., and that ATCC

6872 be reassigned from *C. ammoniagenes* to be included in *C. stationis*. Because these strains alkalize citrate, we propose that the description of the genus *Corynebacterium* be amended to include citrate-positive strains. Although the phenotypic tests used were not an exhaustive list, our results suggest that discrimination between *C. stationis* and *C. ammoniagenes sensu stricto* will be difficult without genetic characterization. Data from this study using phylogenetically characterized strains suggested that *C. stationis* strains differed from *C. ammoniagenes* by pigment (yellow or yellowish colonies, not grey–white after 48 h), by fermentation of mannose and by production of pyrazinamidase by most strains. Discrimination of both species from the taxa suggested by *apiweb* using API Coryne strips, i.e. *C. renale* and *C. pseudotuberculosis*, may be done using tests shown in Table 1.

Emended description of the genus *Corynebacterium* Lehmann and Neumann 1896

Species in the genus exhibit the properties described by Collins & Cummins (1986); in addition, strains of some species also alkalize citrate.

Description of *Corynebacterium stationis* (ZoBell and Upham 1944) comb. nov.

Corynebacterium stationis (sta.ti.o'nis. L. gen. n. *stationis* of a fixed position).

Basonym: *Brevibacterium stationis* (ZoBell and Upham 1944) Breed 1953.

Other synonym: '*Achromobacter stationis*' ZoBell and Upham 1944.

This description is based on the description of *Brevibacterium stationis* given by Jones & Keddle (1986) and from

Fig. 1. Relationship of nearly full-length 16S rRNA gene sequences from strains of *Corynebacterium stationis* comb. nov. and phylogenetically closely related *Corynebacterium* species, with *Corynebacterium diphtheriae* NCTC 11397^T and *Rhodococcus equi* DSM 20307^T as near and far outgroups, respectively. The tree was generated by the neighbour-joining method. Sequences determined in this study are highlighted in bold. Bar, 0.01 substitutions per nucleotide position.

Fig. 2. Relationship of partial *rpoB* gene sequences from strains of *Corynebacterium stationis* comb. nov. and phylogenetically closely related *C. ammoniagenes*, with *C. tuberculostearicum* CCUG 45418^T as an unrelated outgroup. The tree was generated by the neighbour-joining method. Sequences determined in this study are highlighted in bold. Bar, 0.02 substitutions per nucleotide position.

observations in this study for strains ATCC 14403^T and ATCC 6872 and two human blood-culture isolates. Gram-positive, asporogenic, short rods, 0.6–1 µm in diameter, occur singly, in pairs and in 'V' forms and can be club shaped. Non-motile. Not acid-fast. Colonies on blood agar after 24 h are grey-white but become yellowish with age, about 1 mm in diameter and raised, with no haemolysis observed. Facultatively anaerobic, but grows better under aerobic conditions. Poor or no growth under strictly anaerobic conditions. Grows at 25, 35 and 42 °C in air. Not lipophilic. Catalase-positive and oxidase-negative. Acid is produced, albeit slowly, from glucose, fructose and ribose. Fucose found in the API 50CH gallery is also weakly reactive. Weakly reactive for mannose. Xylose, mannitol, lactose, sucrose, maltose, galactose, glycerol, glycogen, raffinose, salicin and trehalose are not reactive. Triple-sugar iron remains neutral or alkaline or neutral. Grows in the presence of 6–10 % NaCl. Urease is produced. Nitrate is reduced to nitrite but nitrite is not reduced to nitrogen using either conventional or API Coryne panel methods. Simmons' citrate is alkalized. Tyrosine is hydrolysed but gelatin, aesculin, casein and starch are not. Not reactive in tests for lysine, arginine or ornithine decarboxylases. Christie-Atkins-Munch-Petersen (CAMP) and CAMP-inhibition reactions are not observed. DNase and indole are not produced. Pyrazinamidase may be produced, but otherwise most enzymes of the API ZYM panel are not detected. Strains are generally susceptible to a wide variety of antimicrobials. The cell-wall peptidoglycan has *meso*-diaminopimelic acid as the major diamino acid. Cell-wall sugars are arabinose and galactose. Mycolates are present. Menaquinones are the sole respiratory quinones, MK-8(H₂) and MK-9(H₂) being detected. CFAs are of the saturated and monounsaturated types and tuberculostearic acid is detected. The DNA G + C content for the type strain is 53.9 mol% (*T_m* method). Members of the species may be discriminated from other *Corynebacterium* species by observation of the alkalization of citrate and other described reactions, and by 16S rRNA and *rpoB* gene sequencing.

The type strain, ATCC 14403^T = CCUG 43497^T = CIP 104228^T = DSM 20302^T = NBRC 12144^T = JCM 11611^T = VKM B-1228^T, was isolated from seawater. Strain ATCC 6872, previously assigned to *C. ammoniagenes*, was isolated

from human infant stools and strains NML 94-0424 and NML 03-0173 were recovered from human blood cultures.

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