Biological and Chemical Bases for the Reclassification of
Brevibacterium vitarumen (Bechdel et al.) Breed (Approved
Lists, 1980) as *Corynebacterium vitarumen* (Bechdel et al.)
comb. nov. and *Brevibacterium liquefaciens* Okabayashi and
Masuo (Approved Lists, 1980) as *Corynebacterium*
liquefaciens (Okabayashi and Masuo) comb. nov.

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On the basis of their biological properties (cell morphology, staining reaction, cellular inclusions, guanine plus cytosine content of deoxyribonucleic acid, genome size, degree of deoxyribonucleic acid homology with *Corynebacterium diphtheriae* [Approved Lists, 1980] strain PW8, pattern of enzymic capacities) and chemical structures (peptidoglycan type, major cell wall polysaccharide, characteristic fatty acids, major phospholipids and glycolipids), two *brevibacteria* are transferred to the genus *Corynebacterium*. *Brevibacterium vitarumen* (Bechdel, Honeywell, Dutcher, and Knutsen) Breed (Approved Lists, 1980) is transferred as *Corynebacterium vitarumen* (Bechdel, Honeywell, Dutcher, and Knutsen) comb. nov., and *Brevibacterium liquefaciens* Okabayashi and Masuo (Approved Lists, 1980) is transferred as *Corynebacterium liquefaciens* (Okabayashi and Masuo) comb. nov.

Although *Brevibacterium vitarumen* does not appear in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (13), this name (10, 14) appears on the Approved Lists, 1980 (45). The organism is of some significance in the history of animal nutrition (8). Recently, it has been shown to produce corynomycolic acids linked as 6,6'-dicorynomycoloyl trehalose and 6-monocorynomycoloyl trehalose (27) and to exhibit other properties which suggest that it might belong in the genus *Corynebacterium* (Approved Lists, 1980 [45]) (24, 51-54). *Brevibacterium liquefaciens* (Approved Lists, 1980 [45]), which is listed in *Bergey's Manual*, 8th ed., was the first microorganism found to excrete cyclic adenosine 3',5'-monophosphate (32, 41). Like *B. vitarumen* it appears to be a *Corynebacterium*. Here we present evidence derived from a study of two strains of *B. vitarumen* and one strain of *B. liquefaciens* and propose that these two *brevibacteria* be transferred to the genus *Corynebacterium*.

MATERIALS AND METHODS

Bacterial strains. We used the following strains in this study: *B. vitarumen* ATCC 10234 (type strain [45]) and strain 12143 (received from the Institute for Fermentation, Osaka, Japan by M.-A.L.) (27); *B. liquefaciens* ATCC 14929 (type strain [45]); *Corynebacterium diphtheriae* (Approved Lists, 1980 [45]) ATCC 27010 (type strain [45]) and PW8 [=PW8,(P); maintained at New York University (26, 40)]; *C. diphtheriae* (*gravis*) strain HF (maintained at New York University [3]); *C. diphtheriae* (*C. ulcerans*) strain 603 (=DLC 603/50; received from S. D. Henriksen [18]); *Corynebacterium equi* ATCC 6939 (type strain [45]); *Corynebacterium flavesens* (Approved Lists, 1980 [45]) ATCC 10340 (type strain [45]) (6); *Corynebacterium pseudodiphtheriticum* (Approved Lists, 1980 [45]) strain H Toulouse (50); *Corynebacterium renale* (Approved Lists, 1980 [45]) ATCC 19412 (type strain [45]); *Corynebacterium xerosis* (Approved Lists, 1980 [45]) strain 53-K-1 (Armed Forces Institute of Pathology) (7); and *Escherichia coli* (Approved Lists, 1980 [45]) ATCC 23724.

Biological and biochemical tests. Methods for assessing the following properties have been described recently (6): (i) presence of polyphosphate (metachromatic) granules, (ii) reduction of potassium tellurite, (iii) catalase activity, (iv) glucan phosphorylase (α -1,4-

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glucan:orthophosphate glucosyl transferase) activity, (v) production of hydrogen sulfide, (vi) deamidation of pyrazinamide, (vii) fermentation of carbohydrates, (viii) hydrolysis of polyoxyethylene sorbitan conjugates, (ix) hydrolysis of sodium hippurate, (x) decarboxylation of ornithine and lysine, (xi) final hydrogen ion concentration in buffered peptone glucose broth, (xii) production of acetoin, (xiii) gelatinase activity, (xiv) nitrate reductase activity, (xv) hydrolysis of urea, (xvi) proteolytic action on milk, (xvii) alkaline phosphatase activity, and (xix) phage susceptibility.

Lipid analyses. Lipid analyses were also performed by methods described recently (1, 2, 6) for (i) the extraction of free lipids, (ii) the fractionation and identification of polar lipids, and (iii) the analyses of fatty acids by thin-layer chromatography and gas-liquid chromatography.

Cell walls. Cell walls were prepared by the method of Asselineau et al. (1). Acid hydrolyses were performed in sealed tubes containing 1 N HCl for sugars and 6 N HCl for amino acids and isomers of diaminopimelic acid. Hydrosoluble components of hydrolysates were studied by descending paper chromatography (Whatman no. 1) in which the following solvent systems were used: butanol-pyridine-water (6:4:3, by volume) for sugars; phenol-ammonia atmosphere for amino acids; and methanol-water-10 N HCl-pyridine (80:17.5:2.5:10) for diaminopimelic acid (9). Sugars were visualized with *p*-anisidine hydrochloride and AgNO₃/KOH; amino acids and diaminopimelic acid were visualized with ninhydrin.

Note that the identifications of the main constituents of the cell walls of corynebacteria were performed both with cell wall preparations as described above and with delipidated cells. We found that the delipidated cells had the same composition as the cell wall preparations with regard to the main sugars, amino acids, and diaminopimelic acid. Therefore, the use of delipidated cells in lieu of cell wall preparations offers an economic and convenient means for differentiating strains quickly after lipid extraction, and the absence of free lipids makes the identification of the cell wall components easier.

Guanine plus cytosine content of DNA, DNA homology, and determination of genome sizes. The preparations of corynebacterial deoxyribonucleic acids (DNAs) for determinations of the guanine plus cytosine contents and for DNA-DNA hybridizations were as previously described (6). The genome molecular weights were assessed from the product of the initial concentration of dissociated DNA (C_0) and the time required for 50% reassociation (the $C_{0t_{1/2}}$ value) of the total DNA, as described by Britten and Kohne (12); see also Seidler and Mandel [43]. DNA from *E. coli* strain ATCC 23724 (genome molecular weight, $\approx 2.5 \times 10^9$) was used as a standard. Thus, genome molecular weight was equal to $(2.5 \times 10^9)(C_{0t_{1/2}} \text{ of DNA})/C_{0t_{1/2}} \text{ of } E. coli \text{ DNA}$.

RESULTS

Analyses of the cell walls of *B. vitarumen* strain 12143, *B. liquefaciens* ATCC 14929 and two reference strains, *C. diphtheriae* strain HF and *C. pseudodiphtheriticum* strain H Toulouse, yielded galactose and arabinose as the major

sugars and glycine, alanine, glutamic acid, and *meso*-diaminopimelic acid as the preponderant amino acids. Thus, the cell walls were characteristic of corynebacteria.

Analyses of the fatty esters of the free lipids from the brevibacteria and the two reference corynebacteria revealed mainly straight chains of 16 and 18 carbon atoms (Fig. 1A through E). There was no evidence for the presence of any branched chains (iso or anteiso), including 10-methylstearic acid (tuberculostearic acid). The pattern of mycolic acids found was that expected in *Corynebacterium*, namely, a predominance of saturated and unsaturated homologs of C₃₂ corynomycolic acids whose esters on pyrolytic

FIG. 1. Separation by gas chromatography of fatty esters from brevibacteria and corynebacteria. (A) *B. vitarumen* strain 12143. (B) *B. vitarumen* ATCC 10234. (C) *B. liquefaciens* ATCC 14929. (D) *C. diphtheriae* strain HF. (E) *C. pseudodiphtheriticum* strain H.

cleavage yielded aldehydes and esters containing 16 and 18 carbon atoms (Fig. 2A through E). Although the corynomycolic acids of *B. vitarumen* strain 12143 contain no double bonds, their profiles after pyrolysis were in general agreement with those of reference corynebacteria (*C. diphtheriae* [*C. gravis*] and *C. pseudodiphtheriticum*), as shown in Fig. 2. The major polar lipids found in the brevibacteria consisted of (i) phospholipids and (ii) glycolipids. The phospholipids included phosphatidyl glycerol, diphosphatidyl glycerol (cardiolipin), phosphatidyl inositol, and small amounts of phosphatidyl inosi-

tomannoside, the latter being detected after chromatography on diethylaminoethyl cellulose. Phosphatidyl ethanolamine was detected in none of the strains examined. These results indicate that the phospholipid pattern for these bacteria is that designated type 1 by Lechevalier et al. (28).

On saponification the glycolipids yielded corynomycolic acids and trehalose. In a study of the glycolipids of *B. vitarumen* strain 12143 (27), two were found, one fast moving and the other slow moving. The former proved to be a dicorynomycolate of trehalose, and the latter was a monocorynomycolate of trehalose. These trehalose mycolates are characteristic products of the *Corynebacterium-Mycobacterium-Nocardia* group of bacteria (2, 22, 44). In the present study dicorynomycolates were found in material from *C. diphtheriae*, *C. pseudodiphtheriticum*, and *B. vitarumen*, and, in addition, monocorynomycolates were found to be associated with *B. vitarumen*. Only monocorynomycolates predominated in extracts of *B. liquefaciens*. Whereas under the conditions of growth used in this study characteristic trehalose glycolipids were found, there are bacteria in the *Corynebacterium-Mycobacterium-Nocardia* group in which the structures of the glycolipids may be markedly influenced by the kind of sugar supplied in the growth medium (11, 23, 37, 46).

Table 1 shows the guanine plus cytosine contents of the DNAs, the extents of homology among the DNAs of strains of *B. vitarumen*, *B. liquefaciens*, and *C. diphtheriae*, and the respective genome molecular weights of these strains.

The strains of both *B. vitarumen* and *B. liquefaciens* consisted of nonmotile, nonspore-forming, gram-positive, non-acid-fast (Ziehl-Neelsen stain), pleomorphic, rod-shaped bacteria exhibiting slightly tapered ends. When grown on phosphate-rich medium, cells contained metachromatic granules which could be revealed with alkaline methylene blue and toluidine blue. Each organism was catalase positive, nitrate reductase positive, and pyrazinamidase positive. Each failed to ferment lactose, to produce ornithine and lysine decarboxylase, to produce an alkaline phosphatase, and to hydrolyze hippurate and sorbitan monooleate. A selection of corynebacteriophages and supernatants from a variety of corynebacteria and related bacteria (6), including strains 12143 and ATCC 14929, failed to form plaques on lawns of *B. vitarumen* 12143 and *B. liquefaciens* ATCC 14929. Supernatants of overnight cultures of *B. vitarumen* strains 12143 and ATCC 10234 showed no plaque-forming ability on lawns of ATCC 10234 and strain 12143.

FIG. 2. Pyrolytic gas chromatography of methyl corynomycolates from brevibacteria and corynebacteria. (A) *B. vitarumen* strain 12143. (B) *B. vitarumen* ATCC 10234. (C) *B. liquefaciens* ATCC 14929. (D) *C. diphtheriae* strain HF. (E) *C. pseudodiphtheriticum* strain H. For differing data, see reference 47. Both temperature of incubation and degree of aeration of culture may quantitatively affect the patterns obtained (16, 47).

TABLE 1. Data relating to the DNAs of *B. vitarumen* and *B. liquefaciens*

Strain	Guanine plus cytosine content of DNA (mol %)	Genome molecular weight ($\times 10^9$) ^a	% Homology with DNA from:	
			<i>C. diphtheriae</i> strain PW8	<i>B. liquefaciens</i> ATCC 14929
<i>C. diphtheriae</i> strain PW8	53.8	1.2	— ^b	64.1
<i>C. renale</i> ATCC 19412	58.2	1.2	34	52.6
<i>B. vitarumen</i> strain 12143	64.8 ^c	1.2	51	68.6
<i>B. liquefaciens</i> ATCC 14929	62.7	1.8	64.1	—

^a Under conditions where the genome molecular weight of a standard preparation of *E. coli* ATCC 23724 is 2.5×10^9 .

^b —, Reference DNA (i.e., interaction should be 100%).

^c A value of 63.3 mol% was obtained by Colwell and Mandel (15).

B. vitarumen strains 12143 and ATCC 10234 grew as raised, smooth, butyrous, yellow colonies on chocolate agar. They grew as black colonies on chocolate tellurite agar and Tinsdale agar. On the latter medium they gave no indication of producing H₂S from cysteine. They produced intracellular starch from glucose 1-phosphate. They fermented glucose, fructose, galactose, maltose, mannose, sucrose, trehalose, and salicin. There was no fermentation of glycerol, inositol, mannitol, or starch. They hydrolyzed and fermented esculin and were methyl red and Voges-Proskauer positive. They effected the hydrolysis of sorbitan monolaurate and urea. Gelatin was not hydrolyzed; milk was neither curdled nor digested, and inspissated serum (as Loeffler slants) was not liquefied by these strains. Division times for strain 12143 were 125 min in PGT medium (6) and 75 min in Trypticase soy broth (BBL Microbiology Systems).

B. liquefaciens ATCC 14929 grew as yellow colonies on Loeffler slants and formed glistening black colonies on chocolate tellurite agar and black colonies with brown halos on Tinsdale agar (i.e., it produced cysteine desulhydrase, as indicated by the production of H₂S in the presence of cysteine and did not produce H₂S from inorganic sulfur sources, such as those in double sugar-iron agar). In contrast to *B. vitarumen*, under standard conditions (6) *B. liquefaciens* fermented none of the above-mentioned sugars, was methyl red and Voges-Proskauer negative, failed to ferment esculin, produced no starch in the presence of glucose 1-phosphate, was urease negative, and failed to hydrolyze sorbitan monolaurate, but it was markedly proteolytic (e.g., it liquefied Loeffler slants, digested milk, and liquefied gelatin). Table 2 shows comparative biochemical patterns of these two species of *Brevibacterium* and corynebacteria. The division time of *B. liquefaciens* ATCC 14929 growing in Trypticase soy broth was 50 min, and in

PGT medium with or without 2% (wt/vol) glucose it was 55 min.

DISCUSSION

On the basis of cell wall composition (peptidoglycans containing *meso*-diaminopimelic acid and the polysaccharide arabinogalactan), mycolic acids, polar lipids, and morphological and biological properties, *B. vitarumen* strains 12143 and ATCC 10234 seem to belong to the same species and to satisfy the generic requirements for *Corynebacterium*. The extent of homology (51%) found between the DNA of strain 12143 and that of *C. diphtheriae* PW8 does not argue against this assignment. The guanine plus cytosine content of strain 12143 agrees with that of the majority of members of *Corynebacterium* (4, 52), and the genome molecular weight is close to that of *C. diphtheriae* (Table 1). Therefore, we transfer *Brevibacterium vitarumen* to *Corynebacterium*, as follows.

Corynebacterium vitarumen (Bechdel, Honeywell, Dutcher, and Knutsen) comb. nov. [Basionym: *Flavobacterium vitarumen* Bechdel, Honeywell, Dutcher, and Knutsen 1928. Synonym: *Brevibacterium vitarumen* (Bechdel, Honeywell, Dutcher, and Knutsen) Breed 1957.]

Generic properties. The cells are nonmotile, nonsporeforming, gram-positive to gram-variable, non-acid-fast (Ziehl-Neelsen stain), pleomorphic, rod-shaped bacteria which exhibit moderately tapered ends (cells from actively growing cultures appear as doublets with tapered ends). When grown on phosphate-rich media, cells contain metachromatic granules which can be revealed by staining with alkaline methylene blue or toluidine blue. In their metabolism they are aerobic to facultatively anaerobic, produce catalase, and fail to ferment lactose. Their cell walls are characterized by peptidoglycans of directly cross-linked *meso*-diaminopimelic acid (A1 γ of Schleifer and Kandler

TABLE 2. Comparative biochemical reactions of *B. vitarumen*, *B. liquefaciens*, and certain species of *Corynebacterium*^a

Strain	Production of:				Esculin hydrolysis			Acid from:				Decarboxylation of:			Hydrolysis of:	
	Pyrazinamidase	Urease	Gelatinase	Acetoin	Cysteine desulfhydrase			Glucose	Maltose	Man-nose	Salicin	Starch	Ornithine	Lysine	Sorbitan mono-oleate	Sorbitan mono-palmitate
<i>C. diphtheriae</i> ATCC 27010	-	-	-	-	+	-	-	+	+	+	-	-	-	-	+	+
<i>C. diphtheriae</i> (<i>C. ulcerans</i>) strain 603	-	+	+	-	+	-	-	+	+	+	-	+	-	-	+	+
<i>C. pseudodiphtheriticum</i> strain H	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	+
<i>C. renale</i> ATCC 19412	+	+	-	+	+	-	-	+	-	+	-	-	-	-	+	+
<i>C. xerosis</i> strain 53-K-1	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+
<i>B. vitarumen</i> ATCC 10234 and strain 12143	+	+	-	+	-	+	-	+	+	+	+	-	-	-	-	+
<i>B. liquefaciens</i> ^b ATCC 14929	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
<i>C. flavescentis</i> ATCC 10340	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	+
<i>C. equi</i> ATCC 6939	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+

^a All of these organisms grow on chocolate tellurite agar (0.01% potassium tellurite).

^b This organism has been reported to utilize certain sugars for growth (31). We found that it is capable of growing in a mineral salts medium with xylose as the sole carbon source.

[42]); the major cell wall sugars are arabinose and galactose (42). The major lipids consist of shorter-chain mycolic acids, diphosphatidyl glycerol, phosphatidyl glycerol, and phosphatidyl inositol.

Specific description of *C. vitarumen*. Originally isolated from bovine rumen, this lemon yellow (on Loeffler slants) *Corynebacterium* exhibits a singular set of biochemical properties, which include formation of black colonies on chocolate agar containing 0.03% potassium tellurite, fermentation of salicin, fermentation and hydrolysis of esculin, hydrolysis of sorbitan monolaurate but not sorbitan monooleate, and production of acetoin, urease, nitrate reductase, and pyrazinamidase. It is not proteolytic. It fails to produce a cysteine desulfhydrase and alkaline phosphatase, does not decarboxylate ornithine or lysine, and does not hydrolyze hippurate. It shares with certain other corynebacteria the capacity to hydrolyze sucrose and trehalose, as well as glucose, galactose, fructose, maltose, and mannose. It is glycerol, inositol, mannitol, and starch negative. It is methyl red positive and produces a starch-like product in the presence of glucose 1-phosphate. Its guanine plus cytosine content is 64.8 mol%, and its genome molecular weight is 1.2×10^9 . Its DNA exhibits 51% homology with the DNA of *C. diphtheriae* PW8 and 68.6% homology with the DNA of *B. liquefaciens* ATCC 14929.

Type strain. The type strain is ATCC 10234 (=NCIB 9291) (45). This is presumed to be the strain on which Bechdel et al. based their original description of *F. vitarumen* (8); a culture of this strain was obtained from Michael A. Farrell, Division of Bacteriology, Pennsylvania State College.

The preceding generic description applies also to *B. liquefaciens*, which, unlike *B. vitarumen*, gave negative fermentation reactions with the various carbohydrates referred to above but did digest inspissated serum (Loeffler slants), milk, and gelatin. *B. liquefaciens* also produces an active cysteine desulfhydrase, as indicated by the halos it formed on Tinsdale agar. When actively growing, *B. liquefaciens* assumes the shape of tapering doublets common to most corynebacteria, but in old (spent) broth cultures, the cells are ovoid to coccal, as, for example, are the cells of *C. pseudotuberculosis* (*C. ovis*). From Table 1 it is evident that *B. liquefaciens* DNA shows 64.1% homology with PW8 DNA and 68.6% homology with the DNA of *B. vitarumen*. Furthermore, it shows 52.6% homology with *C. renale*, suggesting a closer relationship with the DNA of *C. renale* than the relationship shown between *C. diphtheriae* and *C. renale*. It

is our opinion that *B. liquefaciens* should be assigned to *Corynebacterium*. Although the specific epithet *liquefaciens* has been used many times in the past for various corynebacteria, it does not appear in combination with *Corynebacterium* in the Approved Lists of Bacterial Names (45). We propose that *B. liquefaciens* Okabayashi and Masuo (30–36) be transferred to *Corynebacterium* as *C. liquefaciens* (Okabayashi and Masuo) comb. nov.

Corynebacterium liquefaciens (Okabayashi and Masuo) comb. nov. (Basionym: *Brevibacterium liquefaciens* Okabayashi and Masuo 1960.)

Generic properties. The generic properties given above for *C. vitarumen* apply also to *C. liquefaciens*.

Specific properties. Originally "isolated from drainage" and selected for its property of "excreting" nucleotides (33) at a time when there was much interest in the nucleotidic precursors of bacterial cell wall components (17, 39), *C. liquefaciens* produces yellow growth on and dissolution of Loeffler medium. It is a pyrazinamidase-positive *Corynebacterium* (I. T. Sulea, M. C. Pollice, and L. Barksdale, Int. J. Syst. Bacteriol., in press) which fails to ferment the common sugars (see above), to produce acetoin, to hydrolyze esculin or hippurate, to produce alkaline phosphatase, to produce a starch-like material from glucose 1-phosphate, to decarboxylate lysine or ornithine, and to hydrolyze sorbitan monolaurate, sorbitan monooleate, or urea. It liquefies gelatin, digests milk, and produces a cysteine desulfhydrase and a nitrate reductase. It grows in a mineral salts medium containing asparagine (i.e., Sauton medium) or other amino acids (e.g., alanine [36]). It is best known for its adenyl cyclase, which is membrane bound (29), requires Mg^{2+} and pyruvate for activation, and catalyzes the conversion of adenosine triphosphate (or deoxyadenosine triphosphate) to cyclic adenosine 3',5'-monophosphate (or cyclic deoxyadenosine 3',5'-monophosphate) and pyrophosphate (19, 20). Pyruvate stimulates the synthesis of cyclic adenosine 3',5'-monophosphate both in vivo and in vitro. In vitro the adenyl cyclase appears allosterically to accommodate the required pyruvate, and the pyruvate-adenyl cyclase functions catalytically (29). Recently, Takai et al. crystallized the adenyl cyclase from *B. liquefaciens* (48), and they have undertaken studies of the thermodynamics of its interaction with its substrate (25). Although these authors found that pyruvate was required for activity of the crystalline enzyme, they left the mode of interaction between the enzyme and its substrate open to question (49). In a study of the

steady-state kinetics of the adenyl cyclase of *B. liquefaciens* Özer and Scheit suggested that activation by pyruvate involves abolishing "negative cooperativity" between the subunits of the cyclase, with concomitant induction of a better fit of the enzyme for its substrate (38). (The remarkable stimulation which pyruvate has for members of the *Corynebacterium-Mycobacterium-Nocardia* group has been pointed out previously [4, 5]. However, in screening a number of bacteria for pyruvate-stimulable versus pyruvate-nonstimulable adenyl cyclases, Ide reported that the adenyl cyclases of *C. equi* ATCC 6939 and *Nocardia erythropolis* ATCC 4277 fall into a class not stimulated by pyruvate [21].)

Type strain. The type strain of *C. liquefaciens* is ATCC 14929 (45).

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REPRINT REQUESTS

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