Enzyme Electrophoretograms in the Analysis of Taxon Relatedness of *Micrococcus cryophilus*, *Branhamella catarrhalis* and Atypical Neisserias

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SUMMARY

Extracts were prepared from *Micrococcus cryophilus*, several strains of *Branhamella catarrhalis* and *Neisseria* spp. Esterases, NADP-dependent isocitrate dehydrogenase and malate dehydrogenase activities were assayed after electrophoresis of extracts of polyacrylamide gels. Except for *Neisseria perflava* and *N. sicca* which resolved activity bands for the acetate-esterase only, the remaining bacteria exhibited species-specific esterase patterns also for the propionate and butyrate substrates. The multiple esterase patterns from *B. catarrhalis* ATCC25238 were qualitatively and quantitatively different from those of *B. catarrhalis* ATCC23246. This finding and other evidence supports a taxonomic shift of the latter to a species level of that genus. The atypical neisserias *N. caviae* and *N. ovis* appeared to exhibit an intragenetic specificity in their esterase patterns with those from *B. catarrhalis* but not to the other *Neisseria* spp. tested. The malate dehydrogenase patterns from the atypical neisserias and *B. catarrhalis* ATCC23246 were qualitatively similar; however, the patterns of isocitrate dehydrogenase activity were variable for these species. *Micrococcus cryophilus* was distinct in its esterase and dehydrogenase bands, strongly suggesting its taxon unrelatedness to the genus *Branhamella* or the atypical neisserias. Of the enzymes assayed, esterase proved to be the most reliable for taxonomic identifications.

INTRODUCTION

Some members of the genus *Neisseria* are generally accepted as being atypical species with uncertain taxonomic status (Catlin & Cunningham, 1961; LaMacchia & Pelczar, 1966; Baumann, Doudoroff & Stanier, 1968). Among these species *Neisseria catarrhalis* has recently been reassigned as the only representative of the newly created genus *Branhamella* (Catlin, 1970) and designated as *B. catarrhalis*. The other atypical neisserias, *N. caviae* and *N. ovis*, continue to be retained within the genus *Neisseria*. Sleytr & Kocur (1971) advanced the view that the psychrophilic, Gram-negative coccus *Micrococcus cryophilus* is taxonomically related to the atypical neisserias, but especially to *B. catarrhalis*. They based this view on the marked similarities in the cell wall morphology and identical guanine-cytosine (GC) contents of 41 mol % in DNA of these latter species (Boháček, Kocur & Martinec, 1969; Bøvre, Fiandt & Szybalski, 1967). Earlier, Mazenec, Kocur & Martinec (1966) described the cell-wall appearance and cell arrangement characteristic of *M. cryophilus* as peculiar and not like that for the type species *M. luteus*.

Fox & McClain (1974) used comparative polyacrylamide gel electrophoresis of soluble proteins to test the relatedness between *M. cryophilus* and *B. catarrhalis*; the results were inconclusive. The protein patterns from these species were grossly dissimilar and demon-

Table 1. *Bacterial species and strains used*

Species	Strain	Source*
<i>Branhamella catarrhalis</i>	NE11	ATCC (25238)
<i>B. catarrhalis</i>	NE4	ATCC (23246)
<i>Micrococcus cryophilus</i>	B57	ATCC (15174)
<i>Neisseria caviae</i>	GP11	ATCC (14659)
<i>N. ovis</i>	NO1	ATCC (19576)
<i>N. ovis</i>	NO2	ATCC (19575)
<i>N. perflava</i>	PF	AUCC
<i>N. sicca</i>	SC	AUCC

* ATCC, American Type Culture Collection; AUCC, Culture collection, Department of Biology, American University. Strains NE11 and B57 are the type strains of *B. catarrhalis* and *M. cryophilus*, respectively.

strated no discernible intragenetic similarity. In addition, the genetically related species *N. perflava* and *N. sicca* (Kingsbury, 1967) also exhibited species-distinct electrophoretic protein patterns which did not substantiate their intragenetic relatedness. The fact that total soluble protein electrophoretograms could not discriminate intragenetic specificity negated such identification intergenetically.

In the present study the continued analysis of taxon relatedness between *M. cryophilus*, *B. catarrhalis* and the atypical neisserias has been extended to an evaluation of enzyme activity patterns following gel electrophoresis of crude cell extracts. The enzymes assayed were esterase, NADP-dependent isocitrate dehydrogenase and malate dehydrogenase. The esterase electrophoretograms were shown to be valuable taxonomic indicators for these species.

METHODS

Organisms. The bacterial species used and their sources are listed in Table 1.

Cultural conditions, preparation of cell extracts and electrophoretic procedures. The medium and cultivation procedures, the preparation of extracts, polymerization of 7 % polyacrylamide gels and the electrophoretic methods were described by Fox & McClain (1974). The samples applied to the gels contained 100 to 115 μ g protein/0.05 ml. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Enzyme staining. Esterase activity was assayed by soaking the gels, after electrophoresis at 23 to 25 °C for 10 min, in 100 mM-potassium phosphate buffer pH 7.0. Gels were then placed into a reaction mixture containing 15 mg Fast blue RR salt, 0.25 ml of 1 % (w/v) α -naphthyl substrate in 50 % (v/v) acetone, and 12.25 ml distilled water (El-Sharkawy & Huisinigh, 1971a). The substrates used were α -naphthyl acetate, α -naphthyl propionate and α -naphthyl butyrate. The gels were incubated at 23 to 25 °C in the dark. When the amber-stained bands appeared the reaction was stopped by transferring the gels to 7 % (v/v) acetic acid. Controls consisted of reaction mixtures in which the α -naphthyl substrate was replaced with 0.25 ml of 50 % acetone.

NADP-dependent isocitrate dehydrogenase and malate dehydrogenase were assayed at 23 to 25 °C by immersing the run gels into a reaction mixture composed of: 100 mM-tris-(hydroxymethyl) aminomethane (tris)-hydrochloride, pH 8.3, 10 ml; distilled water, 8 ml; NAD (in the case of malate dehydrogenase) or NADP (for isocitrate dehydrogenase), 13 mg; nitroblue tetrazolium chloride (NBT) in 1 ml acetone, 9 mg; phenazine methosulphate (PMS) in 1 ml acetone, 3 mg; and 50 mM DL-malic acid or isocitric acid, as appropriate.

The gels were incubated in the dark. The reactions were terminated after the purple-stained bands developed by placing the gels in 7 % acetic acid. Parallel controls were run using the same reaction mixture except that either the appropriate substrate or cofactor was eliminated (El-Sharkawy & Huisingh, 1971*b*).

RESULTS

Activity staining for esterases and isocitrate and malate dehydrogenases subsequent to electrophoresis of cell extracts on polyacrylamide gels produced band patterns characteristic of stable phenotypes from each of the species tested. Variations occurred in the M_f values (ratio of migration distance of activity band to migration distance of dye front marker) between different runs, although the characteristic band patterns remained unchanged. For a particular species pattern the M_f values were averaged; however, the number of replicate assays was not standardized.

The enzyme electrophoretograms resulting from the three substrates used to assay esterase activity (Fig. 1) reveal specific differences in the overall multiple isoenzyme patterns between *B. catarrhalis* strains ATCC25238, ATCC23246 and *N. caviae* ATCC14659. Though the multiple isoenzymes in strain ATCC25238 and in several other *B. catarrhalis* strains generally appeared to be nonspecific, those from ATCC23246 and ATCC14659 showed a distinct specificity with respect to the acetate substrate. In ATCC23246 the major band at M_f 0.78 of the acetate pattern is probably qualitatively identical with the major bands at M_f 0.76 resolved in both its propionate and butyrate patterns. Similarly, the minor bands at approximately M_f 0.62 and M_f 0.67, occurring in the propionate and butyrate patterns derived from ATCC14659, may be the same activity bands exhibited in its acetate pattern.

The extract prepared from *M. cryophilus* ATCC15174 and *N. ovis* strains ATCC19576 and ATCC19575 were characterized by a single but distinct nonspecific esterase band. In contrast, *N. perflava* (PF) and *N. sicca* (SC) each exhibited a single diffuse activity band for the acetate substrate only.

Isocitrate and malate dehydrogenase activity was not resolved initially from the extracts of all the species following electrophoresis. Strains ATCC15174 and SC were devoid of isocitrate dehydrogenase activity after the electrophoresis of approximately 100 μ g protein, although malate dehydrogenase bands were resolved for both strains at this concentration. In addition, an isocitrate dehydrogenase activity band was resolved in the extract derived from strain PF, yet no malate dehydrogenase band was detected.

Extracts from the species exhibiting no electrophoretically-resolved dehydrogenase activities were assayed spectrophotometrically for NADP-dependent isocitrate dehydrogenase (Thomas, Doelle, Westwood & Gordon, 1972) and malate dehydrogenase (Ochoa, 1955); the dehydrogenase activities were present (Table 2). Consequently, these extracts were re-examined following electrophoresis of increased units of the dehydrogenase. An isocitrate dehydrogenase band was resolved from ATCC15174 after separating 0.07 unit, whereas no band was detected originally at 0.011 unit applied. A faint but definite isocitrate band was exhibited from SC at 0.09 unit applied, but unresolved at 0.027 unit. A minimum of 0.9 to 1 unit of malate dehydrogenase from PF was necessary in order to exhibit an activity band (Fig. 2).

Fig. 1. Schematic diagrams of esterase activity band patterns. Top row: acetate substrate bands. Middle row: propionate substrate bands. Bottom row: butyrate substrate bands. Sequence of species: Ne11 (*B. catarrhalis* ATCC25238); Ne4 (*B. catarrhalis* ATCC23246); GP11 (*N. caviae* ATCC14659); NO1 (ATCC19576), NO2 (ATCC19575) (*N. ovis*); B57 (*M. cryophilus* ATCC15174); PF (*N. perflava*); SC (*N. sicca*).

DISCUSSION

In this study the esterase activity patterns proved reliable taxonomic determinants, more so than those for malate dehydrogenase, and were not at all reliable for isocitrate dehydrogenase. Other investigators have reported on the value of esterase electrophoretograms in the characterization of bacterial groups such as the β -haemolytic streptococci (Lund, 1965),

Table 2. *Specific activities of malate and NADP-dependent isocitrate dehydrogenases*

Species	Enzyme	Specific activity (units of activity*/mg protein)
<i>M. cryophilus</i>	ICDH (NADP)	0.11
	MDH (NAD)	3.15
<i>N. sicca</i>	ICDH (NADP)	0.27
	MDH (NAD)	14.80
<i>N. perflava</i>	ICDH (NADP)	4.65
	MDH (NAD)	1.23

ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase.

* One unit of enzyme activity is the quantity of enzyme catalysing production of 1 μ mol NADPH₂ (NADH₂) per min at 23 to 25 °C.

Fig. 2. Schematic diagrams of isocitrate and malate dehydrogenase activity band patterns. Species codes as in Fig. 1.

corynebacteria (Robinson, 1968), and phytopathogenic bacteria (El-Sharkawy & Huisingh, 1971*a, b*).

The differences in the characteristics of the esterase isoenzyme patterns from ATCC23246 and the type strain ATCC25238 provides additional evidence that ATCC23246 is sufficiently distinct physiologically and in some of its other properties (Catlin & Cunningham, 1964; LaMacchia & Pelczar, 1966; Baumann *et al.* 1968; Fox & McClain, 1974) to warrant its reassignment to the species level. Nevertheless, this fact does not preclude their possible intragenetic relatedness, since similar activity bands at M_f 0.705 in their propionate and

butyrate patterns were resolved from strains ATCC23246 and ATCC25238 despite quantitative differences. This observation, together with the extent of reported genetic transformation frequencies (Bøvre, 1967) between ATCC23246 and ATCC25238 would support the retention of the former as a species of *Branhamella*.

The existence of an esterase activity band at M_f 0.70 to M_f 0.705 in the patterns from the atypical neisserias ATCC19576, ATCC19575 and ATCC14659, and also *B. catarrhalis* strains ATCC25238 and ATCC23246 (excluding its acetate pattern) further advances the view of an identifiable intrageneric specificity among these species. Since the electrophoretograms resolved from ATCC19576 and ATCC19575 were virtually indistinguishable they have been recognized here as a single taxonomic entity (ATCC19576). Based on the comparative acetate-esterase activity patterns from strains PF and SC it would seem appropriate to consider strains ATCC14659 and ATCC19576 as more closely allied to strains ATCC25238 and ATCC23246 than to the former *Neisseria* spp. Additional support of this is provided by evidence from genetic (Henriksen & Bøvre, 1968) and molecular (Kingsbury, 1967) studies.

Our results demonstrated clearly that the esterase activity patterns resolved from the extract of ATCC15174 exhibited no similarity to such patterns from the other species. The idea that a natural taxon relatedness exists between strains ATCC15174 and ATCC25238 or the atypical neisserias strains ATCC14659 and ATCC19576 based on cytological and GC content similarities (Sleytr & Kocur, 1971) is therefore not substantiated from these results, or, as described previously, by a comparison of total soluble protein electrophoretograms (Fox & McClain, 1974).

The slight variations in electrophoretic mobility between the malate dehydrogenase bands from strains ATCC23246, ATCC14659 and ATCC19576 strengthen the possibility for taxonomic relatedness between these species as inferred from their esterase patterns. Moreover, the dissimilarity of the malate dehydrogenase bands from the atypical neisserias compared with those from PF and SC was also consistent with the results obtained from their esterase patterns. This further demonstrates the legitimacy for a taxonomic segregation of these latter species as does the distinct specificity in the malate dehydrogenase band of *M. cryophilus*.

The degree of heterogeneity in M_f values in the isocitrate dehydrogenase patterns (Fig. 2) is similar to that reported by Rowe & Reeves (1971) for several related and unrelated bacterial species and strains; thus isocitrate dehydrogenase electrophoretograms do not assist in identifying taxonomic interrelationships of bacteria.

The initial absence of activity bands of isocitrate dehydrogenase (ATCC15174, SC) and malate dehydrogenase (PF) was not due to inactivation of these enzymes during the preparation of the cell-free extracts (Table 2). These enzymes were unresolved, either due to a function of their low concentration in the protein sample, or as a result of their lability at low concentrations during electrophoresis (Rowe & Reeves, 1971). The resolution of the previously unresolved dehydrogenases was accomplished by increasing three- to tenfold the units of enzyme activity loaded on to the gels. The specific activities of isocitrate dehydrogenase in the extracts from strains ATCC15174 and SC were 30 to 65 times less, respectively, than their malate dehydrogenase activities, whereas the specific activity of isocitrate dehydrogenase from PF was approximately four times that of its malate dehydrogenase activity. These findings explain the initial absence of bands as a result of the electrophoretic separation of enzymes at concentrations below the sensitivity of the assays. However, these results do not provide any evidence which would exclude the possibility that electrophoretic lability might have occurred at low enzyme concentrations.

In these experiments the lower limit of resolution of dehydrogenase activity bands necessitated the electrophoretic separation of 0.06 to 1 unit of enzyme activity. Thus false negative

enzyme electrophoretograms may arise when the separation of proteins is based on total concentration rather than on the endogenous units of specific enzyme.

Our results lead to the following conclusions. The strain presently designated *B. catarrhalis* ATCC23246 is a distinct species and should be reclassified as such within the genus *Branhamella*. *Micrococcus cryophilus* (ATCC15174) is unrelated to *B. catarrhalis* ATCC25238 and the atypical neisserias. It is concluded conditionally that the species *N. caviae* and *N. ovis* should be shifted from the genus *Neisseria* to the genus *Branhamella* as independent species.

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