

Differentiation of *Micrococcus luteus* and *Micrococcus varians* on the Basis of Catalase Isoenzymes

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SUMMARY

Crude extracts prepared from four *Micrococcus varians* strains, 11 *M. luteus* strains and four laboratory isolates subsequently classified with *M. luteus* were assayed for catalase activity following electrophoresis on polyacrylamide gels. The enzyme patterns produced from the *M. varians* strains exhibited three catalase isoenzymes which were distinguished into two types of patterns depending upon the location of the major band. The extracts from all the *M. luteus* strains produced the same pattern, composed of two catalase isoenzymes of similar electrophoretic mobility. For both species the isoenzyme patterns agreed with the differentiation based on biochemical properties. The catalase activity staining method was shown to be a restricted yet reliable assay in the intrageneric but not intraspecies differentiation of yellow-pigmented micrococci.

INTRODUCTION

Currently, the aerobic Gram-positive yellow micrococci are divided into two species in the genus *Micrococcus* (Baird-Parker, 1974). Most of the strains now distributed between these species were previously designated as distinct *Micrococcus* spp., whereas others were classified as species of the genera *Sarcina* and *Staphylococcus*. The species differentiation of these strains is based on the guanine plus cytosine (GC) content in DNA (Boháček, Kocur & Martinec, 1967, 1970; Kocur, Bergan & Mortensen, 1971; Rosypal, Rosypalová & Hořješ, 1966; Rosypalová, Boháček & Rosypal, 1966; Venner, 1967) and on their action on glucose and nitrate. *Micrococcus luteus* (Kocur, Páčová & Martinec, 1972) has a GC content in DNA of 70.7 to 75.5 %, is asaccharolytic and does not reduce nitrates. *Micrococcus varians* (Kocur & Martinec, 1972) has a GC content in DNA of 66 to 72 %, produces acid from glucose, and generally reduces nitrates. Catalase is produced by all strains of both species.

Fox (1975) reported additional evidence supporting the concept of a close phenetic relatedness between *M. luteus* and *M. varians*, based on common intrageneric protein electrophoretic patterns which did not differentiate between representative strains of these species. Therefore, it was of interest to determine whether catalase (hydrogen peroxide oxidoreductase, EC. 1.11.1.6), universally produced by these bacteria, would display differential inter- and/or intraspecific isoenzyme patterns on polyacrylamide gels. This paper describes the catalase activity patterns obtained from 19 strains of yellow micrococci.

Table 1. *Biochemical properties of tested strains*

Species	Designation on receipt	Source‡	Glucose§	Nitrate	Starch	Gelatin	Urease	Catalase
<i>Micrococcus luteus</i>	<i>M. luteus</i> *	ATCC4698	—	—	—	—	+	+
<i>M. luteus</i>	<i>M. flavus</i>	ATCC10240	—	—	+	+	—	+
<i>M. luteus</i>	<i>M. flavus</i> 81	AUCC	—	—	—	—	—	+
<i>M. luteus</i>	<i>M. luteus</i> 1226	AUCC	—	—	+	+	—	+
<i>M. luteus</i>	<i>M. lysodeikticus</i> PH9	AUCC	—	—	—	—	+	+
<i>M. luteus</i>	<i>M. lysodeikticus</i> XL30	AUCC	—	—	—	—	+	+
<i>M. luteus</i>	<i>M. sodonensis</i>	ATCC11880	—	—	—	+	—	+
<i>M. luteus</i>	<i>Sarcina flava</i> 22	AUCC	—	—	—	±	+	+
<i>M. luteus</i>	<i>S. lutea</i>	ATCC381	—	—	—	—	+	+
<i>M. luteus</i>	<i>S. lutea</i> SL233	AUCC	—	—	+	+	+	+
<i>M. luteus</i>	<i>S. subflava</i>	ATCC7468	—	—	+	+	—	+
<i>M. varians</i>	<i>Staph. lactis</i> †	ATCC15306	+	+	—	+	—	+
<i>M. varians</i>	<i>M. varians</i>	ATCC399	+	+	—	+	—	+
<i>M. varians</i>	<i>S. aurantiaca</i>	ATCC146	+	—	+	+	—	+
<i>M. varians</i>	<i>M. pulcher</i>	ATCC15936	+	+	—	—	—	+
		YP02	—	—	—	+	—	+
		YP07	—	—	+	+	+	+
		YP10	—	—	+	+	—	+
		YP15	—	—	+	+	—	+

+, Positive test; —, negative test; ±, weak positive test.

* Neotype strain, *M. luteus*.

† Neotype strain, *M. varians*.

‡ ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; AUCC, Culture Collection, Department of Biology, The American University; YP, strains isolated in this laboratory by R. H. Fox from dust, air and soil.

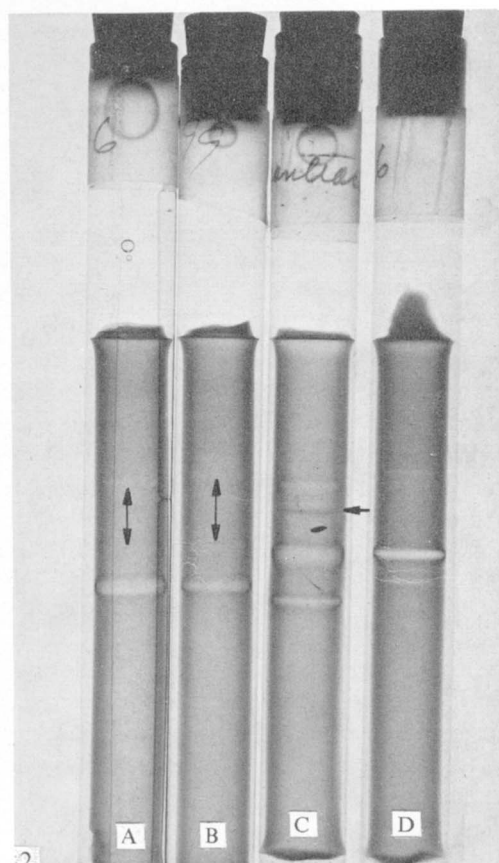
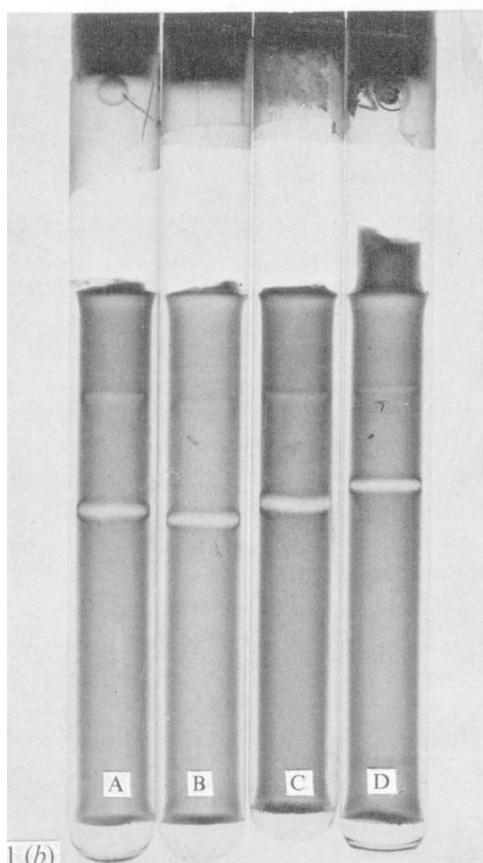
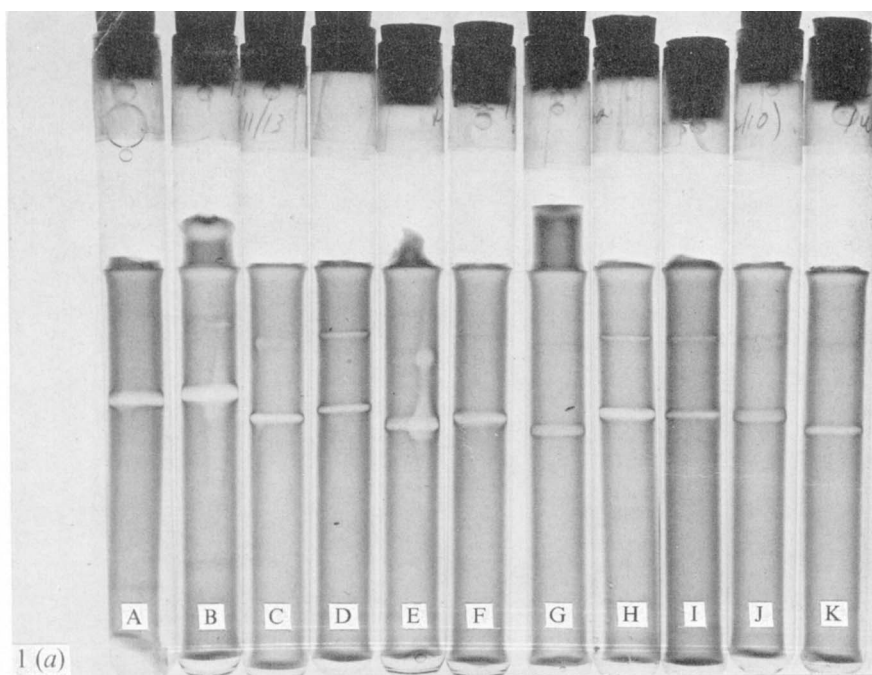
§ Positive test indicates acid from glucose aerobically and anaerobically.

METHODS

Bacteria and cultural conditions. The bacteria tested are listed in Table 1. Cultures for the preparation of crude cell-free extracts were grown in nutrient broth (Difco), with shaking, at 30 °C for 20 to 30 h. The four Gram-positive yellow micrococci isolated in this laboratory were purified by standard techniques from nutrient agar plates inoculated with dust, soil and air from various sources.

Biochemical tests. Acid from glucose was tested with 1 % (w/v) glucose added aseptically to the Hugh & Leifson oxidation–fermentation medium (Difco) with and without a mineral oil seal. Nitrate reduction (0.1 % nitrate broth, Difco), starch hydrolysis (1 %, w/v, soluble starch in nutrient agar, Difco), gelatin liquification (nutrient gelatin, Difco) and urease production (2 %, v/v, urea added aseptically to urea agar base, Difco) were determined by methods described in the 9th edition of the Difco Manual. Catalase was detected by emulsifying growth from overnight cultures on nutrient agar on a glass slide in a drop of 3 % (v/v) H₂O₂ and observing the formation of bubbles.

Preparation of cell-free extracts and electrophoretic procedures. The methods of cell disruption and polymerization of 7 % polyacrylamide gels were described by Fox & McClain (1974). The separating gels were prepared at least one day before use and stored at 4 °C. Samples of 50 µl, containing approximately 250 µg protein, were applied to the gels. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.



Catalase activity stain on gels. Visualization of catalase on gels following electrophoresis was by the diaminobenzidine-peroxidase method of Gregory & Fridovich (1974). Control staining assays consisted of duplicate gels of each sample, one of which was pre-soaked in the mixture from which the peroxidase reagent was eliminated, followed by soaking the gels in the H_2O_2 substrate solution; only peroxidase active bands develop on gels in this assay.

RESULTS AND DISCUSSION

Results of the biochemical tests demonstrated that the *M. luteus* and *M. varians* strains could be distinguished by their actions on glucose and nitrate reduction (Table 1). The reactions of the four isolates indicated that they belonged to the species *M. luteus* (Kocur & Martinec, 1972). Activity in the starch, gelatin and urease tests were strain-variable, and in many instances were at variance with published data (Kocur *et al.*, 1972; Kocur & Martinec, 1972). The deviations in biochemical characters observed here might be explained by the fact that cultural conditions influenced strain instability in the expression of some phenotypes although genotypic loci exist for these characters. Although the laboratory isolates differed from each other in these variable properties, they corresponded biochemically with some of the designated *M. luteus* strains. Thus, it is possible that YP02, YP07, YP10 and YP15 may be identical isolates of already existing strains. Attempts to isolate strains biochemically typical of *M. varians* were unsuccessful.

Electrophoretic separations of cellular proteins from the extracts of *M. luteus* strains (including those from the four isolates) stained for catalase activity, consistently exhibited two isoenzyme bands (Fig. 1*a, b*). With a few minor exceptions, these patterns appeared uniformly similar. In each, the major band was achromatic and broad and of similar electrophoretic mobility, except in AUCC81 in which the major band was slightly more mobile. After staining, the single minor isoenzyme band was visualized either as an achromatic band, generally sharply delineated, or as a chromogenic band as in the patterns from AUCC SL233 and ATCC11880. Gregory & Fridovich (1974) described the appearance of such chromogenic bands as enzyme activity associated with peroxidase or with low levels of catalase. To distinguish between these possibilities, control staining assays were made by preparing gels from the extracts of AUCC SL233 and ATCC11880 and soaking them in the peroxidase-deficient mixture followed by soaking in the H_2O_2 substrate. On these reacted gels neither the major achromatic band nor the minor chromogenic band developed, thus establishing the catalase nature of the latter components. The quality of the minor band from AUCC SL233 and ATCC11880 is probably not different from those of the minor achromatic bands in the patterns from the other *M. luteus* strains, since all these bands had nearly equivalent electrophoretic mobility. This suggests that in AUCC SL233 and ATCC11880 the minor isoenzyme component constituted a quantitatively low but detectable contribution to the total catalase activity (not determined) contained in their respective extracts.

Fig. 1. Catalase isoenzyme patterns from (a) *M. luteus* strains and (b) laboratory isolated strains. (a) (A) *M. luteus* ATCC4698; (B) *M. lysodeikticus* AUCC P119; (C) *M. lysodeikticus* AUCC XL30; (D) *M. flavus* ATCC10240; (E) *M. flavus* AUCC81; (F) *M. sodonensis* ATCC11880; (G) *M. luteus* AUCC1226; (H) *Sarcina subflava* ATCC7468; (I) *S. flava* AUCC22; (J) *S. lutea* ATCC381; (K) *S. lutea* AUCC SL233. (b) (A) YP02; (B) YP07; (C) YP10; (D) YP15. Migration is from top to bottom.

Fig. 2. Catalase isoenzyme patterns from *M. varians* strains. (A) *Staphylococcus lactis* ATCC15306; (B) *M. varians* ATCC399 (vertical arrows show position of minor bands); (C) *S. aurantiaca* ATCC146 (horizontal arrow indicates peroxidase band); (D) *M. pulcher* ATCC15936. Migration is from top to bottom.

In contrast, the enzyme activity patterns produced by extracts of *M. varians* (ATCC15306, ATCC399, ATCC146 and ATCC15936) exhibited three catalase isoenzymes; one major and two minor bands (Fig. 2). In ATCC146 the dense staining band (shown by arrow) proved to be a peroxidase active band. It can be seen that two types of intraspecies pattern developed, which differed in the location of the major band. The patterns from ATCC15306 and ATCC399 were identical, the major band in each being the rapidly-migrating isoenzyme. For ATCC146 and ATCC15936 the major catalase band migrated more slowly and was located unequally between the minor isoenzymes. Whether these differences are limited to the strains examined here or are indeed typical of catalases from other unexamined strains of *M. varians* cannot be evaluated from these few results. Studies have been undertaken to examine this question.

Strain ATCC146 differed from the other *M. varians* strains tested in possessing peroxidase activity, and differed biochemically by its inability to reduce nitrate (Table 1). Although ATCC146 was not included in the redefinition of the taxonomic status of *M. luteus* (Kocur *et al.*, 1972) or *M. varians* (Kocur & Martinec, 1972), it seems appropriate from my results to include this strain with *M. varians*. The reported GC content in DNA of ATCC146 (68 %) is in keeping with this classification (Kocur *et al.*, 1971; Rosypalová *et al.*, 1966). The inability of ATCC146 to reduce nitrate was not considered to be an excluding property since Kocur & Martinec (1972) reported nitrate reduction in 80 % of the *M. varians* strains tested.

The differentiation of *M. luteus* and *M. varians* by their catalase isoenzyme patterns agreed with their differentiation by biochemical tests. *Micrococcus luteus* strains, which do not reduce nitrate or produce acid from glucose, are further characterized by a catalase pattern consisting of one minor and one major band. In comparison, *M. varians* strains, which are generally positive for these properties, possess three forms of the enzyme catalase which can be demonstrated electrophoretically. Further, the four laboratory isolates which were identified as strains of *M. luteus* by biochemical tests exhibited catalase isoenzyme patterns consistent with those characteristic of this species. Despite the limitations of the catalase activity assay as a tool in systematic bacteriology, my results show the usefulness of the method in the genus *Micrococcus* at the intrageneric level. The results also indicate the possibility that the catalase activity assay may be useful in differentiating strains of *M. varians* at the intraspecies level.

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