Pyrazine Carboxylamidase Activity in *Corynebacterium*

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A total of 124 strains representing *Corynebacterium diphtheriae* (*mitis*, *gravis*, *intermedius*, and *ulcerans*) and 12 strains of the closely related *Corynebacterium pseudotuberculosis* (*Corynebacterium ovis*) were found to be pyrazinamidase negative (Pyz⁻). By contrast, a large number of strains representing various corynebacterial species, nocardias, bacterionemas, actinomyces, and rothias were pyrazinamidase positive (Pyz⁺). The usefulness and possible implications of this finding are discussed, and a rapid test for pyrazinamidase activity is described.

In characterizations of microorganisms, negative traits (e.g., inability to hydrolyze certain substrates, to synthesize pigments, or to grow in the presence of oxygen) can be of considerable value. This is especially true if the genetic information governing the reaction(s) responsible for the other (allelic) state (the positive trait or condition) is completely lacking in those bacterial species which exhibit the negative trait. Bacteria which are negative for a given property may have developed from lines that either (i) never carried the genetic information governing the property or (ii) have lost the information via deletion. It is most convenient for classification if such negative organisms in their natural habitats are not likely to acquire the missing information as a result of either conjugation, transformation, or plasmidization.

For the past 3 years we have been searching for new markers with which to characterize the genus *Corynebacterium*. In the course of our screening we discovered that all of the strains of *C. diphtheriae* employed in our laboratory for various studies over a period of years failed to deaminate pyrazinamide. By contrast, strains of *C. xerosis* and *C. pseudodiphtheriticum* did produce pyrazine carboxylamidase, i.e., they were pyrazinamidase positive (Pyz⁺). To test further the prevalence of the pyrazinamidase-negative (Pyz⁻) state of *C. diphtheriae*, we obtained from a variety of sources additional strains of this organism and of the closely related *C. pseudotuberculosis* (*C. ovis*). All of these also proved to be Pyz⁻. On the other hand, a number of other corynebacteria and related organisms were found to be Pyz⁺.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1.

Detection of pyrazine carboxylamidase: hydrolysis of pyrazinamide. Butts of pyrazinamidase test agar were prepared by the method of Wayne (55). This agar contained the following components: Dubos

broth base, 6.5 g in 1,000 ml of quartz-distilled water; pyrazinamide, 0.1 g; sodium pyruvate, 2.0 g; and agar, 15.0 g; the agar was dispensed at 5 ml per screw-capped tube (16 by 125 mm) and autoclaved at 15 lb of pressure for 15 min. These tubes were inoculated with bacteria harvested from overnight growth on single chocolate agar slants (about 5×10^9 bacteria) and with few exceptions were incubated at 37°C overnight. To each butt, 1.0 ml of freshly prepared aqueous ferrous ammonium sulfate (1%, wt/vol) was added. The tubes were examined after 1 to 4 h of refrigeration for the appearance of a pink band in the agar; such a band indicated the presence of pyrazinoic acid. For the rapid pyrazinamidase test, the Dubos broth base was eliminated, and the amount of agar was reduced to 0.2% (wt/vol). Saline suspensions of bacteria which were harvested from single chocolate agar slants were introduced by pipette and mixed in the top one-half of the medium. After incubation for 2 h in a 37°C water bath, 1 ml of freshly prepared reagent was mixed in the top one-half of each tube. Within 1 to 5 min, a pink color appeared in tubes containing positive cultures. Negative reactions necessitated further testing. In establishing the usefulness of the rapid method, we always inoculated fresh pyrazinamidase test agar tubes and incubated them for 7 days in the case of negative reactions. The results were always negative. Thus, the validity of the rapid test seems well established.

RESULTS

All 124 documented strains of *C. diphtheriae*, including varieties *gravis*, *mitis*, *intermedius*, and *ulcerans*, and all 12 strains of *C. pseudotuberculosis* (*C. ovis*) were found to be pyrazinamidase negative. In addition, the single strain of the species from bovine-associated habitats, *C. flavescens*, also failed to deaminate pyrazinamide. In marked contrast, strains of *C. equi*, *C. kutscheri*, *C. minutissimum*, *C. pseudodiphtheriticum*, *C. renale*, and *C. xerosis* were pyrazinamidase positive. In addition, strains of *C. bovis*, *C. cystitidis*, *C. fascians*, *C. genitalium*, *C. paucumetabolum*, *C. pilosum*, and corynebacteria from cases of leprosy, as well as strains of *N. cardiae*, including strains of *N. asteroides*, *N. caviae*, *N. corynebacteroides*, *N. erythropolis*,

TABLE 1. *Strains used in this study*

Species	Strain	Source and/or reference ^a
<i>C. diphtheriae (mitis)</i>	C7 _s (-) Tox ⁻ (=ATCC 27010), C7 _s (β) Tox ⁺ (=ATCC 27012)	5, 6
	A028, P17886, P18011, P21804	27
	A3573	AS
	A8184 N-Red ⁺	28
	A8888, 324 (=F-324), 326 (=F-326)	EIP (48)
	C4 (=C444), 1180	16
	Co443	26
	Lorente, 3203	JMC
	M207, M209	25
	PW8, (P) Tox ⁺	36, 47
	Robb, 324-Sr	This laboratory
	53-A-16	AFIP (8)
	2474, 2478, 2485, 2487, 3310, 3356, 9717, 18004	PM
	B1, B5, B6, B8	WBG (28)
	B3, B12, Harold Bardosen, 1170/Ore	WBG
Nitrate reductase-negative strains (<i>C. belfanti</i>)	106, 107, 108, 109, 113	NBG (24)
	1030 Tox ⁻	AS (42)
	1030 (β) Tox ⁺	2
	3880, 4876, 39191	JMC
	EB79	NYU
<i>C. diphtheriae (intermedius)</i>	NCTC 3987, NCTC 3988, NCTC 5011, NCTC 5012, NCTC 5013, NCTC 5014	NCTC
	NS 810, NS 1057	MF (18)
	4465	AS (51)
	C8, (-) Tox ⁻ (=Halifax flat, HF), C8, (φ) Tox ⁺ (=Halifax)	4
<i>C. diphtheriae (gravis)</i>	A8782	48
	CSIR-4 and CSIR-7 ^b	HRC (13)
	G9, G13, G25, G27, G60, G64, G67, G69	PF (54)
	G27-Sr	This laboratory
	G53-A-7	AFIP (8)
	M77	PF
	NCTC 3985, NCTC 7282, NCTC 7286, NCTC 7289, NCTC 7290, NCTC 7359	NCTC
	NS 832	MF (18)
	R2/53, R13/55, R27/52, R111, R134/54, R135/54, R156	DTR (50)
	SD3-105, SD3-127, SD3-149, SD3-150, SD3-151, SD4-37, SD4-100, SD4-101, SD5-13, SD5-20, SD18, SD19	BED
	4961	JMC
	7508	PM
	CU 170	29
	DLC-Hd 81 (=DLC 1613/51), DLC-Hd 84 (=DLC 1403/51)	DHH (31)
	DLC 603/50, DLC 842/50, DLC 903/50, DLC 1605/50, DLC 1661/50, DLC 1771/50	SDH (29)
<i>C. diphtheriae (ulcerans)</i>	40C, 298G	AS
	976(LZ) Tox ⁺	SBA; this laboratory
	5291, 37142, 39164, 48255, 51166, 51167, 51168, 51169, 52103	JMC
	9304	AS (43)
	ATCC 7715	ATCC
	42 (=ATCC 29593)	RY (59)
	ATCC 6939	ATCC
	ATCC 11890, NCTC 1621, NCTC 7502, 384, 545 ^c	REG
	ATCC 12974	ATCC
	ATCC 12975, ATCC 13000	REG
<i>C. flavescens</i>	ATCC 10340	7
<i>C. genitalium</i>	392-1 type I (=ATCC 33030), 418H type II, 436D type III, 321D type IV, 502F type V	GF (19)
<i>C. genitalium</i> commensals ^d	148A type C-1, 473D type C-2, 261F type C-3, 252B type C-4, 162C2 type C-5, 465 type C-6	GF (19)

TABLE 1—continued

Species	Strain	Source and/or reference ^a
<i>C. kutscheri</i>	ATCC 15677 NCTC 949, NCTC 1386, NCTC 3655 ^c 7813	ATCC NCTC JBN (46)
<i>C. minutissimum</i>	ATCC 23348 NCTC 10284, NCTC 10285, NCTC 10289 66-124	ATCC NCTC MEM
<i>C. paurometabolum</i>	ATCC 8368, ATCC 15530	ATCC
<i>C. pilosum</i>	46 (=ATCC 29592)	RY (59)
<i>C. pseudodiphtheriticum</i>	ATCC 10700, ATCC 10701 EB80, Katz 7, 8, 28, 87, 377, 504, 823, 1788, 7743 153	ATCC This laboratory AS (45) JMC
<i>C. pseudotuberculosis</i> (<i>C. ovis</i>)	ATCC 19410 A, LM, 21 CSIR-1, CSIR-2, CSIR-5 CSIR-6 (Z) Tox ⁺ NCTC 4681, NCTC 4683, NCTC 4691 32244	ATCC AS (43) HRC (13) 2 NCTC JMC
<i>C. renale</i>	ATCC 10849, ATCC 19412 NCTC 7449	ATCC NCTC
Received as <i>C. renale</i> type I	ATCC 10848, FS113/63, H25, R/4, 8, 9, 71, 88 (D-720)	RY (35, 58)
Received as <i>C. renale</i> type II	Mukai 2, Saitama 1, Sasaki, 14 (NU1361), 45, 92 (RH), 119 (Sa-20)	RY (35, 58)
Received as <i>C. renale</i> type III	D731, 44 (Nagahama), 47 (Tojyo), 104, 77 (D-454-1), 772/ 66	RY (35, 58)
<i>C. xerosis</i>	ATCC 373, ATCC 7094, ATCC 7711, ATCC 9016 ATCC 809, ATCC 12319/ Xerosis NCTC 7243, NCTC 9755 53-K-1 78 ^g	ATCC ATCC NYU NCTC AFIP (8) AS (45) 11
<i>Corynebacterium</i> sp. (iso- lated from leprosy pa- tients)	Medalle X Moreno, 43 ^b 2628 LB, 2628T60 D32, D40, FPSA, L3, L4, L11, 84, 86, 273 Kim 1-A	This laboratory 11, 17 JD (38) LK
Anomalous pyrazinami- dase-positive, nonfer- menting corynebacterial strains	NCTC 7238, NCTC 7883, NCTC 7929 NCTC 8481 ⁱ 245 ^j	NCTC NCTC JMC
<i>Actinomyces israelii</i>	Israelii	NYU
<i>A. naeslundii</i>	398	AM (15)
<i>A. viscosus</i>	ATCC 15987, ATCC 19246 A1351	ATCC AM (15)
<i>Bacterionema matruchoi</i>	ATCC 14265, ATCC 14266	ATCC
<i>Brevibacterium vitaru-</i> <i>men</i>	ATCC 10234 12143	ATCC 37
<i>B. liquefaciens</i>	ATCC 14929	ATCC
<i>Nocardia asteroides</i>	ATCC 9969, ATCC 10905, ATCC 14759 (three colony form mutants), ATCC 19247, CDC 287, GUH-2, GUH- 5, Mahvi	BLB
<i>N. caviae</i>	ATCC 14630, CDC 112	BLB
<i>N. corynebacteroides</i>	S	53
<i>N. erythropolis</i>	ATCC 4227	BLB
<i>N. kirovani</i>	1374	TI
<i>N. pelligrino</i>	UWO-4 (=ATCC 15998)	BLB
<i>N. restrictus</i>	AY-B226	CV (49)
<i>N. rhodochrous</i>	ATCC 370	BLB
<i>N. serratia</i>	181	BLB

TABLE 1—continued

Species	Strain	Source and/or reference ^a
<i>Nocardia</i> sp.	A403 [*]	34
<i>Nocardia tenuis</i>	ATCC 15907	ATCC
<i>Rhodococcus rhodochrous</i>	372 (=ATCC 13808)	REG
<i>Rothia dentocariosa</i>	ATCC 17931	ATCC

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; AFIP, U.S. Armed Forces Institute of Pathology; AM, A. Munzenmaier; AS, A. Saragea, Cantacuzino Institute, Bucharest, Romania; BED, B. E. Diamond, State Department of Health, Pierre, S.D.; BLB, B. L. Beaman, University of California School of Medicine, Davis; CV, C. Vezina; DHH, D. H. Howard; DTR, D. T. Robinson; EIP, E. I. Parsons; GF, G. Furness; HRC, H. R. Carne; JBN, J. B. Nelson; JD, J. Delville; JMC, J. M. Coffey, New York State Department of Health, Albany; LK, L. Kato, Université de Québec, Laval-des-Rapides, Québec, Canada; MEM, M. E. McBride, Baylor College of Medicine, Houston, Tex.; MF, M. Frobisher, Jr.; NBG, N. B. Groman; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London NW9 5HT, England; NYU, Collection of the Department of Microbiology, New York University School of Medicine, New York; PF, P. Frederiq, Department of Microbiology and Hygiene, University of Liege, Liege, Belgium; PM, P. Maximescu, Cantacuzino Institute, Bucharest, Romania; REG, R. E. Gordon, Rutgers, The State University of New Jersey, Piscataway; RY, R. Yanagawa; SDH, S. D. Henriksen; TI, T. Imaeda, College of Medicine and Dentistry of New Jersey, Newark; WBG, W. B. Gundersen, State Institute for Public Health, Oslo, Norway. SBA, S. B. Arden.

^b Received as *C. ovis*.

^c Received as *Mycobacterium rhodochrous* (*C. equi*).

^d Now designated *C. pseudogenitalium* (20).

^e Received as *C. murium* (*C. kutscheri*).

^f Received as *C. pseudotuberculosis*.

^g Received as *C. hofmannii*.

^h Isolated from recent cases of leprosy.

ⁱ Received as *C. xerosis*.

^j Received as *C. ovis*.

^k Received as *Mycobacterium friburgensis*, susceptible to nocardiphages.

N. pelligrino, and *N. rhodochrous*, were all pyrazinamidase positive. The anomalous strains NCTC 7238, NCTC 7883, NCTC 7929, NCTC 8481 (received as *C. xerosis*) and 245 (received as *C. ovis*), as well as strains of *Bacterionema matruchotii*, *Rothia dentocariosa*, *Actinomyces israelii*, *Actinomyces naeslundii*, and *Actinomyces viscosus*, were also pyrazinamidase positive.

DISCUSSION

The 124 strains of *C. diphtheriae* examined are representative of a vast number of strains involved in outbreaks of diphtheria and/or isolated in surveys for *C. diphtheriae*. For example, the *gravis*, *mitis*, and *intermedius* strains isolated by the Leeds investigators (1, 44), some of which now bear American Type Culture Collection and/or National Collection of Type Cultures accession numbers, were obtained from a study of 25,000 cultures derived from as many cases of diphtheria. The examples of *C. pseudotuberculosis* (*C. ovis*) used by us are representative of a number of ovine infections associated with a well-characterized etiological agent (*C. pseudotuberculosis*). If the genomes of these strains carried the information required for synthesis of a pyrazine carboxylamidase, an occa-

sional *Pyz*⁺ mutant should have been found as an unselected marker among the members of this large sample. The fact that such was not the case is taken to indicate that *C. diphtheriae* and *C. pseudotuberculosis* (*C. ovis*) lack the genetic information for the synthesis of corynebacterial pyrazinamidase; i.e., they are *Pyz*⁻. We have elsewhere designated the *Pyz*⁻ corynebacteria as group 1 (6). These corynebacteria share additional properties which distinguish them from such *Pyz*⁺ species as *C. pseudodiphtheriticum*, *C. xerosis*, *C. renale*, and representatives of *Nocardia* and *Mycobacterium*. They share a requirement for pantothenic acid (or β -alanine) (2). They all produce a neuraminidase which cleaves *N*-acetylneuraminic acid residues from animal mucins, gangliosides, and sialyl-lactose (3). Furthermore, they synthesize an *N*-acetylneuraminic acid lyase which splits *N*-acetylneuraminic acid into its components, *N*-acetylmannosamine and pyruvic acid. Under suitable conditions this same lyase in test tubes combines *N*-acetylmannosamine with pyruvate to form *N*-acetylneuraminic acid. This property is absent from other members of *Corynebacterium* and from representatives of *Mycobacterium* and *Nocardia* (3). Another property shared by Group 1 corynebacteria is the capacity to give expres-

sion to the gene *tox*, which is carried by certain corynebacteriophages. When any group 1 corynebacterium is lysed or lysogenized by a *tox*-carrying corynebacteriophage, the singular protein diphtherial toxin is synthesized (5). Until recently (6), the members of group 1 have been treated as three distinct species, primarily on the basis of habitat (12, 51, 56). *C. diphtheriae* (*gravis*, *mitis*, and *intermedius*) was associated with human throats, *C. ulcerans* was associated with cattle, and *C. pseudotuberculosis* (*C. ovis*) was associated with lymphangitis and other chronic, purulent infections of sheep, goats, and horses (12). Actually, the range of hosts suitable for the growth of *C. diphtheriae* (*ulcerans*) includes humans, cows, and horses (21, 22, 43), and when it has been looked for in human populations, the variety *ulcerans* seems to have been found in most parts of the world (14, 29–32, 43, 52). *C. pseudotuberculosis* (*C. ovis*) has been isolated from sheep, goats, buffaloes, horses (13, 43), and humans (10). Although no broad search has been made for *C. diphtheriae* in domestic animals, some strains received by us as *C. pseudotuberculosis* (*C. ovis*) from veterinary bacteriologists are indistinguishable from *C. diphtheriae* (*gravis*). Furthermore, animal infections with *C. diphtheriae* have been recorded (23, 41).

The only other known Pyz⁻ corynebacterium is *C. flavesens*. The type strain of this species was isolated from cheese, and other isolates have been reported from a cow stall, milking instruments, and cow dung (57). Since only the type strain of *C. flavesens* exists, one cannot assume that all strains of this organism are negative. The group 1 corynebacteria and *C. flavesens* seem to have links to ungulates, particularly the bovine species. Since ungulate species were on the earth long before humans, they must have been colonized by the ancestors of corynebacteria of group 1, and on their mucous membranes, *C. diphtheriae* (*ulcerans*) and *C. pseudotuberculosis* (*C. ovis*) evolved. During domestication of the ungulate species, humans became available for colonization. Suggestions of a close relationship among the three have been indicated from time to time. For example, microbiologists working on surveys related to outbreaks of diphtheria have described corynebacteria which were like *C. diphtheriae* in some respects and like *C. pseudotuberculosis* (*C. ovis*) in others (9, 32, 33, 39, 40, 43). An outright search for more such examples should be made. Meanwhile, studies of existing strains with regard to genome size, deoxyribonucleic acid homology, and fatty acid profiles should indicate whether the varieties of *C. diphtheriae* and *C. pseudo-*

tuberculosis (*C. ovis*) represent a single bacterial species.

For diagnostic bacteriologists concerned with screening organisms from human oropharynxes, the pyrazinamidase status of a pleomorphic, gram-positive, unknown organism could be useful in the following way. If a candidate for identification as a strain of *C. diphtheriae* is isolated, it should be pyrazinamidase negative, whereas the morphologically confusing *C. pseudodiphtheriticum* (*C. hoffmanii*) and *C. xerosis* should be pyrazinamidase positive. Other pleomorphic, gram-positive organisms from oropharynxes include *B. matruchotii*, *R. dentocariosa*, *A. naeslundii*, *A. viscosus*, and *A. israelii*. All of these are Pyz⁺. The fact that this test can be done within 2 h (using suitable numbers of bacteria) makes it a test of considerable value for diagnostic laboratories.

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REPRINT REQUESTS

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