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Diphthericin types, bacteriophage types and serotypes of *Corynebacterium diphtheriae* strains isolated in Australia

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SUMMARY

A diphthericin typing scheme has been constructed using 441 strains of *Corynebacterium diphtheriae* isolated in eastern Australia from 1962 to 1971. Ten types have been distinguished using seven strains of *C. diphtheriae* and two strains of *C. belfanti* as indicators of the diphthericins produced by the newly isolated strains. Strains grouped into types L2, L3 and L3*a* were found only in Melbourne and types L1 and L4 were predominant in Sydney. Type L5 strains were isolated intermittently throughout the period of study and were found in all eastern states. Numerical analysis of the characteristics of the strains suggests that associations exist between, on the one hand, diphthericin type and, on the other hand, bacteriophage type, serotype and biochemical activity.

INTRODUCTION

The typing of *C. diphtheriae* by means of surface antigens (Ferris, 1950) or by sensitivity to bacteriophages (Saragea & Maximescu, 1966, 1969) has been in use for a number of years. It has been known for more than 20 years (Thibaut & Welsch, 1949; Thibaut & Fredericq, 1956) that *C. diphtheriae* produces bacteriocins and it was therefore decided to establish a typing scheme using these diphthericins. While the work was being carried out, independent reports of bacteriocin activity among strains of *C. diphtheriae* appeared from Russia (Emelyanov, Musonova & Lavnik, 1968) and Romania (Meitert, 1969). It was hoped that diphthericin typing would be useful with those strains which could not be typed by serological means or by their sensitivity to bacteriophages (Gibson, Cooper, Saragea & Maximescu, 1970).

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MATERIALS AND METHODS

Source of strains

The strains isolated in Victoria or received from New South Wales have been described previously (Gibson *et al.* 1970). South Australian strains were forwarded to the Microbiological Diagnostic Unit (M.D.U.) from the Institute of Medical and Veterinary Science in Adelaide, while the strains isolated in the Northern Territory, near Darwin, and those isolated in Cairns, Queensland, were received from the School of Public Health and Tropical Medicine in the University of Sydney.

Bacteriological techniques

The biochemical, serological, bacteriophage-typing and virulence tests were described previously (Gibson *et al.* 1970), but a few additions and amendments were made. The ability to liquefy gelatin was examined by growing the strain, for 14 days at 37° C., on a Loeffler slope upon which an Oxoid charcoal gelatin disk had been placed. The technique of Cook (1950) was employed for nitrate reduction using a strip of sterile filter paper, soaked with a 5 % solution of potassium nitrate, on horse blood agar. In this way six strains, together with one positive control strain of *C. diphtheriae* and one negative control strain of *C. belfanti*, could be tested on each plate.

Rabbit antisera containing glycerol were used for slide agglutination and, if a reaction was observed with any antiserum, the organism was retested in 76·0mm. × 13·0 mm., rounded-bottomed, glass test-tubes by adding an equal volume of a suspension of live organisms (equivalent to the 'Wellcome' opacity tube no. 2, Burroughs Wellcome and Co., London) to doubling dilutions of antiserum not containing glycerol. A titre of 1/160, or more, was recorded as significant.

In outline, the method of Gillies (1964), employed for *Shigella* strains, was used to determine the diphthericin activity of strains of *C. diphtheriae* and to determine the bacteriocin activity of other organisms in relation to the diphtheria bacillus. The strain being tested for production was inoculated as a thin diametric streak across freshly prepared Oxoid tryptone soya agar containing 5 % horse blood, and was incubated at 30° C., for 48 to 72 hr. The growth was scraped off and the remaining cells killed with chloroform vapour. A modification of the apparatus of Wahba & Lidwell (1963) was used for strains being tested for sensitivity. They were applied to the surface of the medium by a holder carrying ten stainless steel plates. Thin strips of filter paper, each soaked in a broth culture of a different strain, were placed in the grooves of a sterile templet into which the stainless steel plates fitted. After the edges of the stainless steel plates had been seeded, they were applied to the medium at right angles to the original line of growth. The cultures were incubated for 36 to 48 hr., at 30° C. or 37° C., and the results recorded.

RESULTS

Bacteriocin activity among the corynebacteria

The scheme of identification described by Cowan & Steel (1965) was followed for all strains of corynebacteria isolated but, in addition, strains of *C. belfanti* were distinguished from strains of *C. diphtheriae* by their failure to show definite reduction of nitrate, by their inability to kill guinea-pigs and by their failure to react with *C. diphtheriae* antisera. It should be mentioned that Gundersen (1959) observed serological cross-reactions between his strains of *C. belfanti* and *C. diphtheriae*. Strains of *C. ulcerans* were not encountered during the ten year period of study.

In general, amongst the corynebacteria tested, only *C. murium* and *C. hofmannii* produced bacteriocins reacting against *C. diphtheriae*, except those of *C. diphtheriae* itself. Bacteriocins of *C. diphtheriae* reacted against *C. belfanti* alone, as well as, of course, *C. diphtheriae*. Eighty-three strains of six species were tested for possible bacteriocin activity against 50 strains of *C. diphtheriae*. The latter strains were selected so that at least one representative of each serotype and of each bacteriophage type (or sensitivity pattern) found in Australia was examined. If the strains of *C. belfanti* (12 strains), *C. xerosis* (17 strains), *C. renale* (6 strains) and *C. bovis* (13 strains) produced bacteriocins then they were not active against our strains of *C. diphtheriae*. However, all five strains of *C. murium* were active against the three representatives of serotype Bennett and the four representatives of serotype Wagland used in these experiments. In addition, the 30 strains of *C. hofmannii*, isolated at the M.D.U., displayed two patterns of activity against the 50 diphtheria strains. The members of one group were active against all strains of *C. diphtheriae*, except those of serotype McLean, and the members of the other group were active against all diphtheria strains except those of serotype McLean and non-virulent strains of serotype 2 and serotype Nadjarian.

The same 50 strains of *C. diphtheriae* were tested for their production of bacteriocins (diphthericins) against the same 83 strains of corynebacteria. Activity was detected only against strains of *C. belfanti* and the pattern of activity is exemplified by the action against *C. belfanti* strains F3517 and F10157 (Table 1). Some strains of the diphtheria bacillus inhibited both strains of *C. belfanti*, some were active against one or other and some inhibited neither. These two strains of *C. belfanti* were therefore incorporated in the diphthericin typing scheme.

The diphthericin typing scheme

As a result of cross-testing 441 strains of *C. diphtheriae* and a few strains of *C. belfanti* isolated in Victoria, New South Wales, South Australia, Northern Territory and Queensland, since 1962, together with a few strains isolated previously, 10 diphthericin types have been distinguished (Table 1). Diphthericin production was chosen as the basis for the typing scheme, rather than diphthericin sensitivity, because the former system distinguished strains which behaved similarly when tested for sensitivity to these agents. Moreover, it seems that production is the more stable characteristic (Abbott & Shannon, 1958). Ten patterns of activity have been distinguished using nine indicator strains and comprise what can be

Table 1. *Diphthericin types of* C. diphtheriae *strains in Australia*

Producer type	Indicator (sensitive) strains*								
	Ferris† Nadjarian	Ferris Wagland	Ferris Bennett	F10157‡	F3517‡	1281§	201742‖	116888‖	Ferris McLean
L1	+ +	+ +	+ +	+ +	+ +	+ +	+ +	—	+ +
L2	—	+ +	+ +	+ +	+ +	+ +	+ +	—	—
L3	—	—	—	+ +	+ +	+ +	+ +	—	—
L3a	+ +	+ +	—	+ +	+ +	+ +	+ +	+ +	—
L4	—	—	—	—	+ +	+ +	—	—	—
L5	—	—	—	—	—	+ +	—	—	—
L6	—	—	—	—	+ +	—	—	—	—
L7	—	—	+ +	+ +	+ +	+ +	—	—	—
L8	+ +	+ +	+ +	+ +	—	—	+ +	—	+ +
LU	—	—	—	—	—	—	—	—	—

* + + Denotes growth inhibited; the diphthericin types of these strains are described in the text.
† Ferris (1950) stock serotypes.
‡ M. D. U. isolates identified as *C. belfanti*.
§ Strain of *C. diphtheriae* received from Dr Saragea.
‖ M. D. U. isolates of *C. diphtheriae*.

described as eight true types, one provisional type (L3a) and one type (LU) in which it is convenient to bring together those strains in which the ability to produce diphthericin was not demonstrated.

An example of type L1 is the indicator strain, M.D.U. strain no. 116888, and an example of type L2 is the Ferris (1950) stock strain of serotype Nadjarian. The indicator strains, Ferris (1950) stock serotypes Wagland and Bennett were generally inactive with respect to diphthericin production, although the former sometimes displayed activity against the indicator strain *C. diphtheriae* 1281 and the latter was occasionally active against *C. belfanti* strain F3517. The indicator diphtheria strain 1281 was received from Dr Alice Saragea and Dr Paula Maximescu and is the only strain which is sometimes capable of displaying activity against itself. All other isolates conformed to the rule that a producer strain was inactive against members of its own diphthericin type.

The indicator diphtheria strain 201742 was diphthericin type L5 and the Ferris (1950) stock strain of serotype McLean was diphthericin type LU. Moreover, the 27 strains which comprised type LU were not only all inactive with respect to diphthericin production, but were also resistant to the action of these agents including the bacteriocins of *C. murium* and *C. hofmannii*. Further, these strains were difficult to type by bacteriophage methods because they were either completely resistant to all bacteriophages in the set, or were sensitive only to bacteriophages 11 and 23 (bacteriophage type VI). The Ferris (1950) stock strain of serotype McLean was also sensitive only to bacteriophages 11 and 23 but differed from other strains of this diphthericin type and bacteriophage type since it was inhibited by the diphthericins produced by strains of diphthericin type L1 and the strain of diphthericin type L8.

Type L3a is provisional because the pattern of activity, shown in Table 1,

Table 2. *Distribution and occurrence of the diphthericin types in Australia*

Diphthericin type	No. of strains	Location	Year of isolation
L1	58	Sydney	1967–8
	15	Elmore and Swan Hill	1967
	1	Melbourne	1969
L2	98	Melbourne	1962–5, 1967, 1969
L3	15	Melbourne	1970
L3a	4	Melbourne	1970
L4	36	Sydney	1963–4, 1967, 1968
	9	Melbourne	1968, 1969, 1970
	3	Swan Hill	1967, 1968
	1	Lismore	1968
L5	91	Melbourne	1962–4, 1967–70
	33	Sydney	1963, 1967, 1968, 1971
	7	Swan Hill	1967
	6	Lismore	1968
	23	Adelaide	1969, 1970
	5	Darwin	1970
	7	Cairns	1972
L6	1	Melbourne	1970
L7	1*		
L8	1	Sydney	1971
LU	20	Melbourne	1964, 1971
	5	Lismore	1968
	2	Sydney	1971

* Hewitt (1947) strain of starch-fermenting serotype 1.

occurred after 24 hr. incubation but, after 72 hr., the pattern changed to that of type L3 since the growths of the indicator diphtheria strains Ferris Nadjarian, Ferris Wagland and 116888 had completely traversed the original diametric streak.

Occurrence of the diphthericin types in Australia

The distribution and occurrence of the diphthericin types in Australia is presented in Table 2. The majority of type L1 strains were detected in Sydney in the latter half of 1967 and throughout 1968; 15 strains were isolated in Central and Northern Victoria in mid-1967, but only one strain of this diphthericin type was found in Melbourne. In contrast, strains of type L2 were restricted to Melbourne and were isolated throughout the period between 1962 and 1965, in 1967 when one strain was found, and in November 1969 when 16 strains were isolated. Similarly, strains of types L3 and L3a were limited to Melbourne, but these were cultured during one period only, in January 1970, in the restricted environment of a mental hospital.

Type L4 strains were well established in New South Wales, in particular at Sydney and Lismore, and occurred in Sydney intermittently throughout the period of study. Type L5 strains were isolated in all eastern states of Australia and

have occurred at regular intervals during the 10 years, particularly in Melbourne and Sydney; approximately 40 % of the strains displayed this pattern of diphthericin production. An example of type L7 has not been isolated in Australia and only one isolate of each of the types L6 and L8 were obtained in Melbourne and Sydney respectively.

Comparison of diphthericin type with other typing characteristics

A numerical analysis of the characteristics of the strains was compiled by Miss Betty Laby and Professor E. J. Williams for Victorian isolates and was applied by the authors to include strains found in other eastern states of Australia. Comparison of diphthericin type with sensitivity to the bacteriophages numbered 1 to 24, with antigens detected at a titre of 1/160 or more, and with biochemical activity is presented in Table 3.

It can be seen that, in general, strains of a given diphthericin type fall into very few bacteriophage types and this is seen most strikingly with diphthericin type L2 in which 97 of 98 strains belonged to one bacteriophage type. However, not all strains of this diphthericin type were bacteriophage type XVI; one strain was sensitive only to bacteriophage 19 and the Ferris (1950) stock strain of serotype Nadjarian, classed as diphthericin type L2, exhibited the bacteriophage sensitivity pattern of type XIV similar to the Hewitt (1947) strain of serotype 1.

Resistance to bacteriophages 1–24 was common among diphthericin type L5 strains; 131 were completely resistant and all but one strain were sensitive to no more than four of the bacteriophages. This one strain, sensitive to bacteriophages 13, 14, 15, 16, 17 and 18, was the single strain of serotype Wallis obtained in 1964 and it is interesting that the Ferris (1950) stock strain of serotype Wallis and four strains of this serotype isolated in 1960 were also characterized as diphthericin type L5 and were sensitive to the same six bacteriophages.

Of the 74 diphthericin type L1 strains, 64 possessed the antigen 6387-Greenwood and five strains were autoagglutinable but were isolated during two outbreaks in which all the other strains were of serotype 6387-Greenwood. Ninety strains out of a total of 98 which were diphthericin type L2 possessed one or both of the antigens Nadjarian and 2 and, again, the five autoagglutinable strains were isolated from one outbreak involving strains of serotype Nadjarian. All isolates of diphthericin type L3 and the provisional type L3a were classed as serotype 6387-Greenwood. Of the 27 isolates of diphthericin type LU, one was serotype 6387-Greenwood, one was serotype Edmonston, 23 possessed antigen McLean and two were autoagglutinable but were associated with an outbreak in which strains of serotype McLean were isolated. As mentioned previously, the Ferris (1950) stock strain of this serotype was classed as diphthericin type LU.

Strains grouped into diphthericin types L4 and L5 possessed a wide range of antigens and two strains, isolated near Darwin, which were placed into type L5 produced even suspensions yet failed to react with any of the typing antisera. Although a wide range of antigens was found in types L4 and L5, it is notable that strains possessing the antigen Johnson were placed in one or other of these two diphthericin types.

Table 3. *Comparison of diphthericin type with other parameters used for typing* C. diphtheriae *strains isolated in Australia*

Diphthericin type	Bacteriophage type, or sensitivity	Antigenic* type	Biochemical† activity m	s	v	No. of strains isolated
L1	XIVC	6387-Greenwood	+	+	+	2
	XIVC	6387-Greenwood	+	−	+	48
	XIVC	6387-Greenwood	−	−	+	7
	8, 9, 10, 11.	6387-Greenwood, 2	+	+	+	1
	Resistant	6387-Greenwood, 2	−	−	+	6
	Resistant	McLean, 2	−	−	+	5
	Resistant	Untypable	−	−	+	5
L2	XVI	Nadjarian	+	+	+	56
	XVI	Nadjarian, 2	+	+	+	16
	XVI	2	+	+	+	4
	XVI	6387-Greenwood	+	+	+	3
	XVI	6387-Greenwood, 2	+	+	+	13
	XVI	Untypable	+	+	+	5
	19.	2	+	+	+	1
L3	XVI	6387-Greenwood	−	+	+	7
	3, 10.	6387-Greenwood	−	+	+	3
	Resistant	6387-Greenwood	−	+	+	5
L3a	Resistant	6387-Greenwood	−	+	+	3
	Resistant	6387-Greenwood	−	−	+	1
L4	XIVC	Nadjarian, Johnson	+	−	+	6
	XIVC	Nadjarian, Johnson	−	−	+	18
	XIVC	Nadjarian	−	−	+	9
	XIVC	Untypable	−	−	+	1
	XIVC	Untypable	+	−	+	1
	3, 10.	Johnson	−	−	+	2
	11.	Johnson	−	−	+	1
	11.	Johnson, 2	−	−	+	1
	Resistant	Johnson	−	−	+	1
	Resistant	Johnson, 2	−	−	+	1
	Resistant	6387-Greenwood	−	+	+	3
	Resistant	6387-Greenwood	−	−	+	2
	Resistant	6387-Greenwood, 2	−	−	+	1
	Resistant	Untypable	−	−	+	2
L5	IV	6387-Greenwood	−	−	+	11
	11, 18.	Johnson	+	−	+	3
	11, 13, 18, 19.	Untypable	−	−	+	6
	13, 14, 15, 16, 17, 18.	Wallis	+	−	−	1
	4, 5, 10.	Johnson	−	−	+	20
	Resistant	2, Johnson	−	−	−	2
	Resistant	2, 6387-Greenwood	−	−	−	1
	Resistant	2, Nadjarian	−	−	−	5
	Resistant	2, McLean	−	−	−	17
	Resistant	2, McLean	+	−	−	1
	Resistant	2	−	−	−	5
	Resistant	2	+	−	−	3
	Resistant	Johnson	+	−	+	21
	Resistant	Johnson	−	−	+	8

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Table 3 (*cont.*)

Diphthericin type	Bacteriophage type, or sensitivity	Antigenic* type	Biochemical† activity m	s	v	No. of strains isolated
L5	Resistant	Nadjarian	+	−	+	9
	Resistant	Nadjarian	−	−	+	33
	Resistant	Nadjarian	−	−	−	4
	Resistant	Nadjarian, 2	+	−	+	3
	Resistant	Nadjarian, Johnson	+	−	+	1
	Resistant	Nadjarian, Johnson	−	−	+	1
	Resistant	Nadjarian, Johnson	−	−	−	1
	Resistant	6387-Greenwood	−	+	+	3
	Resistant	6387-Greenwood, 2	+	+	−	1
	Resistant	Untypable	−	−	+	7
	Resistant	Untypable‡	−	−	−	5
L6	Resistant	6387-Greenwood, 2	+	−	−	1
L7	XIV	1	+	+	+	1
L8	XIVC	6387-Greenwood	+	−	+	1
LU	VI	McLean	+	−	+	13
	Resistant	McLean	+	−	−	5
	Resistant	McLean	−	−	−	1
	Resistant	McLean, 2	−	−	+	4
	Resistant	6387-Greenwood	+	−	−	1
	Resistant	Edmonston	+	−	+	1
	Resistant	Untypable	+	−	−	2

* In those strains which carried two antigens, the antigen listed first was detected at a titre of 1/1280, or more; the other antigen was detected at a titre of 1/160 to 1/640. However, 10 of the 16 strains identified as diphthericin type L2 and serotype Nadjarian/2 reacted equally with both of the respective antisera to a titre of 1/1280, or more.

† m+ = acid from maltose; s+ = acid from starch; v+ = virulence for the guinea-pig.

‡ Even suspensions of two of these strains failed to react with any of the typing antisera. All other untypable strains were autoagglutinable in suspension.

The strains identified as *C. diphtheriae* were not homogeneous in the properties of maltose fermentation, starch hydrolysis and ability to kill guinea-pigs, and the non-random distribution of these properties between the different diphthericin types is detailed in Table 3. It is notable that the 56 non-virulent strains were confined to the types L5, L6 (one strain) and LU. Diphthericin type L2 was found to be composed of strains that fermented maltose and starch, and killed guinea-pigs. It was mentioned above that nearly all members of this diphthericin type also belonged to one bacteriophage type, yet the strains were isolated over a relatively long period of time (Table 2).

DISCUSSION

When the methods of typing *Corynebacterium diphtheriae* are compared, then 94 % of the strains isolated in eastern Australia were typed by the diphthericins they produced and, in the remaining 6 %, diphthericin production was not detected.

In comparison, 92 % of the isolates were typed by serological methods and, when the bacteriophages numbered 1 to 24 were used, 50 % of the strains fell into well-established bacteriophage types, a further 10 % displayed irregular patterns of sensitivity to these agents and the remaining 40 % could not be typed by the bacteriophages of this set, although a few strains were placed into two new types using bacteriophages in addition to those mentioned above (Gibson *et al.* 1970).

The strains which could not be typed on the basis of diphthericin production were grouped into a type which might be heterogeneous and it may be necessary to obtain strains from other countries in order to construct types for them. That this might be a fruitful line of work was indicated by strains grouped into diphthericin type L5 since they were distinguished by their action on *C. diphtheriae* strain 1281 received from Romania. At present type L5 appears widespread in Australia, but, as more indicator strains are found, it may be possible to subdivide it into further types. The existence of subtypes within diphthericin type L5 is indicated by the high degree of variation in biochemical activity, by the many bacteriophage types and by the many serotypes. However, one subtype might be composed of the 34 strains which were bacteriophage-resistant, did not ferment starch, were non-virulent and possessed major antigen 2; a second subtype might be composed of the 20 strains which were serotype Johnson and sensitive to bacteriophages 4, 5 and 10; and a third subtype might contain the 11 strains which were serotype 6387-Greenwood and bacteriophage type IV.

It is notable that diphthericin types L1 and L4 were found mainly in New South Wales and that the types L2, L3 and L3a were restricted to Melbourne. The epidemiological factors involved in the association of one diphthericin type with one location are not known, but another interesting facet of this study is that those New South Wales strains which were grouped into bacteriophage type XIVC (Gibson *et al.* 1970) were placed into either diphthericin type L1 or L4 and those Victorian strains classed as bacteriophage type XVI (previously called X–XVI–XVIII, Saragea & Maximescu, 1969) were all grouped into diphthericin type L2.

A second, little-understood group of associations emerging from this study are the relationships between diphthericin type and some of the other bacteriological characteristics used to identify the diphtheria bacillus. One well-documented 'classical' variety of *C. diphtheriae* possesses the ability to ferment maltose and starch, and is virulent for the guinea-pig. Nearly all such strains that were isolated came from Melbourne, belonged to diphthericin type L2, to the bacteriophage type XVI and varied only in the antigens possessed. The chronological appearance of these antigenic variants is interesting; strains with antigen Nadjarian appeared in 1962, 1963 and 1964, and, towards the end of 1964, strains serotyped as Nadjarian/2 were isolated. In March of the following year, the five serotype 2 strains were found. Two years later, a single strain of serotype Nadjarian/2 was obtained. Finally in 1969, one outbreak was caused by strains of serotype 6387-Greenwood/2 and strains of the highly specific serotype 6387-Greenwood. It is tempting to postulate that these strains are all descendants of a single,

parental strain which, during the 10-year-period, had undergone some form of 'antigenic drift'.

The strains which did not produce detectable amounts of diphthericin were virtually all of serotype McLean and those strains which were untypable by serological or bacteriophage methods were grouped into diphthericin type L5; hence, two or more independent methods of typing should be used in investigating the spread of strains in any outbreak of diphtheria.

Strains representative of some of the diphthericin types, serotypes and bacteriophage types described in this paper, together with the diphtheria indicator strains used for the diphthericin typing scheme, have been deposited with Professor McEntegart at the University of Sheffield, England, and the two indicator strains of *C. belfanti*, F10157 and F3517, have been deposited with the National Collection of Type Cultures, Colindale, London, being N.C.T.C. no. 10837 and N.C.T.C. no. 10838 respectively.

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