Differentiation of Nocardioform Actinomycetes by Lysozyme Sensitivity

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INTRODUCTION

Modern taxonomic methods have helped to clarify the relationships between actinomycetes that reproduce by the fragmentation of all, or more or less accidentally involved parts, of their hyphae into bacillary and coccoid elements (Goodfellow & Minnikin, 1977; Mordarska, 1977). Improved classification provides a framework for recognizing and developing tests of value for identification. Simple and reliable tests are particularly required for the identification of mycolic acid-containing bacteria with a wall chemotype IV that are classified in the genera *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* (Goodfellow, 1973; Goodfellow & Minnikin, 1978). Related actinomycetes received as *Gordona aurantiaca* and as *aurantiaca*-like strains are in need of further study to clarify their taxonomy (Goodfellow & Alderson, 1977; Goodfellow *et al.*, 1978).

The sensitivity of actinomycetes to lysozyme has been studied frequently (Romano & Sohler, 1956; Sohler *et al.*, 1958) and is used in the classification and identification of mycobacteria, nocardiae and related strains (Gordon, 1966; Gordon *et al.*, 1974; Lechevalier & Lechevalier, 1974; Gordon & Barnett, 1977). The lysozyme (lytic) test described below provides a convenient way of distinguishing nocardiae, mycobacteria and *G. aurantiaca* strains from related bacteria.

METHODS

Strains. Two hundred representatives of the genera *Actinomadura*, *Corynebacterium*, *Micromonospora*, *Micropolyspora*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Pseudonocardia*, *Rhodococcus* and *Streptomyces* and related strains were studied (Table 1). Cultures were maintained on glucose yeast extract agar at room temperature (Gordon & Mihm, 1962).

Lysozyme test. Strains were grown in submerged cultures in glucose yeast extract broth at 30 °C (Prauser & Falta, 1968). At stationary phase, 5 ml amounts of broth were centrifuged and the pellets were resuspended in sterile distilled water after grinding the organisms where necessary. The suspensions were diluted with distilled water, to give an absorbance (0.2 to 0.25) corresponding to that of an *Escherichia coli* suspension containing about 3×10^8 cells ml⁻¹, and transferred to calibrated test tubes. To 0.7 ml of suspension, 0.4 ml of a 1% (w/v) aqueous solution of freshly prepared Bacto-lysozyme (Difco) was added, and the mixture was incubated for 3 h at 37 °C; 0.7 ml of a 20% (w/v) aqueous solution of sodium dodecyl sulphate (SDS, Sigma) was then added. After thorough mixing, the turbidity of the suspension was compared with that of control suspensions. In each series of tests the controls included known lysozyme-resistant and -sensitive strains. A negative control was also prepared by incubating 7 ml of bacterial suspension with 0.4 ml distilled water for 3 h at 37 °C and then adding 0.7 ml distilled water in place of the SDS solution. The SDS solution can be stored at room temperature but should be warmed before use, to dissolve the SDS completely, and then allowed to cool to room temperature.

Nocardia amarae, *N. asteroides*, *N. autotrophica* and *Rhodococcus erythropolis* strains that were either not lysed or only partially lysed were re-tested using cells defatted with ethanol/diethyl ether (1:1, v/v) or chloroform/methanol (2:1, v/v).

RESULTS AND DISCUSSION

By means of the lysozyme test, nocardioform and related bacteria can be divided into three groups. Thus, strains more or less completely lysed were scored positive, those showing no or only a small degree of lysis were scored negative, while suspensions showing an absorbance of about half that of the initial suspension were considered to be partially lysed (Table 1).

The lysozyme-resistant group is composed mainly of *G. aurantiaca* strains, mycobacteria (with the exception of *M. album*) and true nocardiae, i.e. *N. asteroides*, *N. brasiliensis*, *N. carnea*, *N. otitidis-caviarum* and *N. transvalensis* (Goodfellow & Minnikin, 1977). *Mycobacterium album* is currently listed as a species *incertae sedis* (Runyon *et al.*, 1974). It is one of a few fast-growing mycobacteria resistant to 200 mg isonicotinamide hydrazide ml⁻¹ (Orlean *et al.*, 1978) and its classification needs further study. Further systematic work is also required to find a niche for the *G. aurantiaca* strains, which form a taxon equivalent to, but clearly separate from, *Mycobacterium*, *Nocardia* and *Rhodococcus* (Goodfellow *et al.*, 1978), and to determine their relationship to lysozyme sensitive *aurantiaca*-like bacteria. The sensitivity or partial sensitivity of *N. autotrophica* strains provides additional evidence for distinguishing them from true nocardiae. *Nocardia autotrophica* strains have a wall type IV but unlike true nocardiae they lack mycolic acids and possess high proportions of iso and anteiso fatty acids (Goodfellow & Minnikin, 1977; Kroppenstedt & Kützner, 1978).

With few exceptions the representatives of the genera *Corynebacterium*, *Micropolyspora*, *Nocardioides*, *Oerskovia*, *Pseudonocardia*, *Rhodococcus* and *Streptomyces* were lysozyme-sensitive and could thereby be readily distinguished from nocardiae, mycobacteria and *G. aurantiaca* strains. In all cases, organisms extracted with the organic solvents behaved like undefatted cells.

The finding that lysozyme resistance is a characteristic property of most nocardiae, mycobacteria and *G. aurantiaca* strains is of interest both in the differentiation of these organisms from related bacteria and in the context of the mode of action of the enzyme. The lysozyme test should be useful in clinical laboratories for the differentiation of mycobacteria and nocardiae from corynebacteria and rhodococci since it limits the number of taxa to be considered in the preliminary identification of nocardioform bacteria. The test also provides a convenient way of distinguishing true nocardiae from streptomycetes and may prove to be useful in distinguishing *Actinomadura* species (Table 1). The method might also be of value in determining the sensitivity of bacteria to lysozyme prior to the extraction of DNA and RNA for reassociation assays.

The results of the present study are in general agreement with those of Gordon & Barnett (1977) who examined the sensitivity of nocardiae, mycobacteria and related actinomycetes to lysozyme. However, the two lysozyme tests are different and the results also show some differences; for example, Gordon & Barnett (1977) found that all *N. autotrophica*, *A. das-sonvillei* and *A. pelletieri*, and most *M. phlei* and *M. smegmatis*, strains were sensitive to lysozyme. The method described here has been shown to separate important genera and has the additional advantage that it can be carried out relatively quickly. However, both methods have advantages and disadvantages and should be applied to a selected collection of strains to determine their reproducibility and differential power.

The differences in the sensitivity of actinomycetes to lysozyme presumably reflect structural differences in the wall. Lysozyme acts on the glycan moiety of the peptidoglycan which in most actinomycetes is *N*-acetylated muramic acid, but in true nocardiae, mycobacteria and micromonosporae the muramic acid is *N*-glycolylated (Azuma *et al.*, 1970; Kanetsuna & San Blas, 1970; Vilkas *et al.*, 1970; Michel & Bordet, 1976). It is premature to

Table 1. Sensitivity of the test strains to lysozyme

Taxon	No. of strains*	Result			Taxon	No. of strains*	Result		
		+	±	-			+	±	-
<i>Actinomadura dassonvillei</i>	5	3	2		<i>N. brasiliensis</i>	3			3
<i>A. madurae</i>	2	2			<i>N. carnea</i>	2			2
<i>A. pelletieri</i>	3			3	<i>N. marina</i>	1			1
<i>A. pusilla</i>	1	1			<i>N. otitidis-caviarum</i>	2			2
<i>A. roseoviolacea</i>	1		1		<i>N. transvalensis</i>	2			2
<i>A. verrucosospora</i>	1			1	<i>Nocardioides albus</i>	4	4		
<i>Corynebacterium diphtheriae</i>	2	2			<i>N. luteus</i>	2	2		
<i>C. glutamicum</i>	1	1			<i>Oerskovia turbata</i>	2	2		
<i>C. hoagii</i>	1	1			<i>Pseudonocardia thermophila</i>	1	1		
<i>C. pseudodiphtheriticum</i>	1	1			<i>Pseudonocardia</i> spp.	3	3		
<i>C. xerosis</i>	2	2			<i>Rhodococcus bronchialis</i>	2	2		
<i>Gordona aurantiaca</i>	8			8	<i>R. coprophilus</i>	2	2		
<i>aurantiaca</i> -like strains	9	7	2		<i>R. corallinus</i>	3	3		
<i>Micromonospora chalicea</i>	1		1		<i>R. equi</i>	8	8		
<i>Micromonospora narashino</i>	1		1		<i>R. erythropolis</i>	10	5	2	3
<i>Micromonospora parva</i>	1		1		<i>R. rhodnii</i>	3		2	1
<i>Micromonospora</i> spp.	3	3			<i>R. rhodochrous</i>	4	4		
<i>Micropolyspora faeni</i>	3	3			<i>R. rubropertinctus</i>	5	5		
<i>Micropolyspora fascifera</i>	1	1			<i>R. ruber</i>	27	27		
<i>Micropolyspora rectivirgula</i>	1	1			<i>R. terrae</i>	2	2		
<i>Mycobacterium album</i>	1	1			<i>Rhodococcus</i> spp.	6	6		
<i>M. aurum</i>	1			1	<i>Streptomyces albus</i>	1	1		
<i>M. borstelense</i>	1		1		<i>S. cellulo flavus</i>	1	1		
<i>M. bovis</i>	1		1		<i>S. citreus</i>	1	1		
<i>M. diernhoferi</i>	1		1		<i>S. fradiae</i>	1	1		
<i>M. flavescens</i>	1		1		<i>S. globisporus</i>	1	1		
<i>M. parafortuitum</i>	1		1		<i>S. griseostramineus</i>	1	1		
<i>M. phlei</i>	2		2		<i>S. griseus</i>	1	1		
<i>M. smegmatis</i>	3		3		<i>S. malachiticus</i>	1	1		
<i>M. thermoresistibile</i>	1		1		<i>S. olivaceus</i>	2	2		
<i>M. tuberculosis</i> H37Ra	1		1		<i>S. venezuelae</i>	1	1		
<i>Nocardia amarae</i>	9	3		6	<i>S. violaceorectus</i>	1	1		
<i>N. asteroides</i>	15			15	<i>S. viridifaciens</i>	1	1		
<i>N. autotrophica</i>	7	2	4	1					

+, Complete or almost complete lysis; ±, partial lysis, approximately 50 % decrease in absorbance; -, little or no lysis.

* Strain designations and histories are available from the authors on request.

relate lysozyme sensitivity in actinomycetes solely to the presence of *N*-acetylated muramic acid and lysozyme resistance to the presence of the *N*-glycolylated type, especially since it has been demonstrated that in other bacteria the sensitivity of the peptidoglycan lytic enzymes can be influenced by other wall components (Mosser & Tomasz, 1970). The influence of wall components other than the peptidoglycan may explain the sensitivity of some of the micromonosporae to lysozyme. Uchida & Aida (1977) have devised a micromethod for differentiating between *N*-glycolyl and *N*-acetyl wall types, which may help to elucidate the basis of the lytic test.

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