Enzymatic and Immunological Characterization of the *Mycobacterium fortuitum* Complex

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The arylsulfatase isozymes of *Mycobacterium fortuitum*, *M. peregrinum*, *M. chelonae* subsp. *chelonae*, and *M. chelonae* subsp. *abscessus* were examined to determine the isozymal and immunological relationship among the members of the *M. fortuitum* complex. Cell extracts were subjected to electrophoresis on agarose and polyacrylamide gel, and arylsulfatase activity was localized using β -naphthyl sulfate as substrate. Unique zymograms were produced for *M. fortuitum*, *M. peregrinum*, and *M. chelonae* which were characteristic for each species. The immunological relationship among the sulfatases was assayed by using immunodiffusion and immunoelectrophoresis followed by sulfatase staining for the enzyme. One of the isozymes of *M. fortuitum* and *M. peregrinum* cross-reacted, showing immunological identity. Antisera to sulfatases of *M. fortuitum* and *M. peregrinum* did not react with sulfatases of *M. chelonae*. The characterization of sulfatase isozymes in extracts of organisms in the *M. fortuitum* complex suggests the division of the *M. fortuitum* complex into two species, *M. fortuitum* and *M. chelonae*, with subspecies designations.

The *Mycobacterium fortuitum* complex consists of a heterogeneous group of rapidly growing acid-fast bacilli that are potentially pathogenic. The complex consists of three species: *Mycobacterium chelonae* subsp. *chelonae* and subsp. *abscessus*, *M. fortuitum*, and *M. peregrinum* (14). Despite the number of studies (9, 17, 18) reported on the *M. fortuitum* complex, questions involving the taxonomic status of the species still exist. Electrophoresis of esterase enzymes (1, 11) and thin-layer chromatography of mycobacterial lipids (7), as well as agglutinin absorption (7, 13, 15), immunodiffusion (16), and immunoelectrophoresis (3, 19), have been utilized independently in efforts to identify and classify these organisms. Because these pathogenic species are the only rapid growers producing large quantities of the enzyme arylsulfatase, it was decided to investigate this enzyme as a possible aid in distinguishing the species within the complex. Enzyme activity demonstrated by differential staining was combined with immunological methods to identify antigenic differences among the arylsulfatase isozymes of the species.

MATERIALS AND METHODS

Cultures. Fourteen strains of organisms within the *M. fortuitum* complex were obtained from three sources: the Trudeau Mycobacterial Culture Collection, Trudeau Institute Inc., Saranac Lake, N.Y.; the New York State Mycobacteriology Laboratory, Al-

bany, N.Y.; and the Erie County Laboratory, Buffalo, N.Y. (Table 1). Three of the Trudeau cultures, representing type strains of their respective species, were supplied to the Trudeau Collection by the American Type Culture Collection and the National Collection of Type Cultures. Cultural and biochemical characteristics were monitored throughout the study as a check for strain mutation or contamination.

Preparation of extracts. All strains were grown in Proskauer-Beck medium (Difco Laboratories, Detroit, Mich.) and incubated at either 30 or 37°C until a dense pellicle had formed on the surface of the medium, at about 2 to 6 weeks, depending on the species. Bacilli were harvested by centrifugation, and the pellet was resuspended in an equal volume of 0.9% sodium chloride. An extract was prepared by subjecting the suspension to ultrasonic disruption using the Sonifier Cell Disruptor model 140 (Heat Systems-Ultrasonic Inc., Plainview, N.Y.). The disruption was performed in an ice bath by using 3-min intervals of sonification for a total of 12 min at 20 W. The disrupted suspension was centrifuged at $14,840 \times g$ for 30 min at 4°C, and the supernatant extract was passed through a 0.2- μ m membrane filter (Millipore Corp., Bedford, Mass.) to remove any remaining viable organisms. Total protein concentration of the cell extract was determined by the biuret method (5). Aliquots of each extract were stored at -70°C until tested.

Electrophoresis. Agarose electrophoresis was carried out on photographic glass plates (8 × 10 cm) overlaid with 15 ml of 1% agarose in barbital buffer (pH 8.2, 0.05 M). The electrophoretic run was carried out at 4°C for 90 min with a potential of 4 to 5 V/cm. With the exception of these modifications, the method

TABLE 1. *Mycobacterial strains examined*

Source	Strain no.	Species
Trudeau Mycobacterial Culture Collection	1529 ^a	<i>M. fortuitum</i>
	1530	<i>M. fortuitum</i>
	1547 ^a	<i>M. peregrinum</i>
	1545	<i>M. peregrinum</i>
	1544 ^a	<i>M. chelonae</i> subsp. <i>chelonae</i>
	1524	<i>M. chelonae</i> subsp. <i>chelonae</i>
	1537	<i>M. chelonae</i> subsp. <i>chelonae</i>
	1542	<i>M. chelonae</i> subsp. <i>abscissus</i>
New York State Mycobacteriology Laboratory	4412	<i>M. chelonae</i> ^b
	4901	<i>M. chelonae</i> ^b
	4902	<i>M. chelonae</i> ^b
	3582	<i>M. chelonae</i> ^b
Erie County Laboratory	5208	<i>M. fortuitum</i>
	5256	<i>M. fortuitum</i>

^a Type strain.^b Subspecies not identified by source.

was essentially that described by Grabar (6). Polyacrylamide gel electrophoresis was performed by the methods of Davis (4) and Maurer (10) using glass tubes (5-mm ID). The stacking gel consisted of 12 mm of 3% acrylamide (pH 6.7) layered over 62 mm of 7% separating gel (pH 9.3). A sample extract of 50 μ l was applied to the stacking gel, and the tubes were placed in a discontinuous buffer system of tris(hydroxymethyl)aminomethane (Tris)-glycine (pH 8.5) and tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.07). Electrophoresis was carried out at room temperature with 4 mA/gel until a bromophenol blue tracking dye reached within 3 mm of the bottom of the gel (ca. 55 min).

Staining. The sulfatase substrate, potassium 6-benzoyl-2-naphthyl sulfate, yields a purple insoluble dye when hydrolyzed and coupled to diazo blue B. The substrate was dissolved in a minimal volume of *N,N*-dimethyl formamide (20 mg in 1 ml) at room temperature. As soon as the substrate was completely dissolved, it was quickly added to 10 ml of a sodium acetate-acetic acid buffer (pH 5.0, 0.2 M). Diazo blue B (10 mg) was dissolved in another volume of buffer (10 ml), and the two solutions were combined, yielding a final concentration of 1 mg of substrate and 0.5 mg of diazo blue B for each ml of buffer solution. The staining solution was prepared each time directly preceding its use. After electrophoresis in agarose or polyacrylamide, gels were incubated immediately in staining solution at 37°C for 2 to 3 h. Immunoassays were washed for 3 days in sodium chloride solution (0.15 M) at 4°C and dried before staining.

Antisera. Antisera to the sulfatases were produced by immunizing male New Zealand white rabbits with the mycobacterial extracts in incomplete Freund adjuvant or saline. Quantities consisting of 0.5 or 1.0 ml of each extract were emulsified with an equal volume of adjuvant. At other times, 0.5 or 1.0 ml of extract in an equal volume of NaCl solution (0.15 M) was used

for immunization. Injections were given every 4 weeks. The initial injection consisted of 2 ml of the extract-adjuvant mixture administered intradermally in the hind footpads and front flanks near the axillary lymph nodes. The second injection consisted of 1 ml of extract-adjuvant mixture given intradermally in multiple sites on the neck and back, and 1 ml of extract-saline mixture given intramuscularly in the hind flanks. The third injection consisted of 2 ml of the extract-adjuvant mixture administered intradermally in multiple sites on the neck and back. Throughout the immunization, trial bleedings were obtained from the rabbits. Each serum was titrated in immunodiffusion versus an aliquot of the same mycobacterial extract used for the immunization. The immunodiffusion plates were washed, dried, and stained for sulfatase activity. Additional booster injections, if necessary, consisted of 2 ml of the extract-saline mixture administered intramuscularly in the hind flanks until a titer of 4 or greater was obtained.

Immunological assays. Immunodiffusion was carried out in 1% agarose in sodium chloride (0.15 M) by the method described by Ouchterlony (12). For immunoelectrophoresis, the method described by Grabar (6) was employed with the following modifications: glass plates were overlaid with 1% agarose in barbital buffer (pH 8.2, 0.05 M), and the electrophoretic run was carried out with 4 to 5 V/cm for 90 min. In situ absorptions in immunodiffusion were performed by the methods described by Crowle (2). For both immunoassays, the plates were washed in sodium chloride solution (0.15 M) at 4°C for 3 days before being dried and stained.

RESULTS

Agarose electrophoresis. Arylsulfatase zymograms resulting from agarose electrophoresis of mycobacterial extracts demonstrated three distinct patterns corresponding to: (i) *M. chelonae*, (ii) *M. fortuitum*, and (iii) *M. peregrinum* (Fig. 1). Each species produced two bands possessing sulfatase activity, all of which migrated anodally from the point of origin. Extracts of eight strains of *M. chelonae* possessed both the fastest (*S*₅) and slowest (*S*₁) migrating bands and could therefore be easily distinguished from the extracts of *M. fortuitum* and *M. peregrinum*. The faster bands (*S*₄) of *M. fortuitum* and *M. peregrinum* extracts had similar electrophoretic mobilities. However, *M. fortuitum* could be distinguished from *M. peregrinum* on the basis of the mobility of the slower band of each species (*S*₃ and *S*₂). The slower band (*S*₃) in strains of *M. fortuitum* migrated more anodally and appeared to be present in higher quantities than did the slower band (*S*₂) of *M. peregrinum*. The sulfatase zymograms were consistent for each species irrespective of: (i) number of strains of each species tested, (ii) number of extracts prepared from each strain, (iii) range of protein concentration for the extracts, and (iv) age of the cultures (Table 2).

FIG. 1. Sulfatase isozymes from agarose electrophoresis of extracts of *M. chelonae* (1), *M. fortuitum* (2), and *M. peregrinum* (3).

TABLE 2. Extracts used for characterization of sulfatases

Species	No. of strains	No. of extracts	Range of protein concn (mg/ml)	Range of culture age (days)
<i>M. chelonae</i>	8	13	0.9–12.3	7–49
<i>M. fortuitum</i>	4	9	2.1–5.0	7–28
<i>M. peregrinum</i>	2	6	1.9–3.6	15–49

Polyacrylamide electrophoresis. The sulfatase zymograms from polyacrylamide electrophoresis revealed more bands than were detected by agarose electrophoresis. All of the sulfatase bands migrated anodally from the origin through the 3% stacking gel and were detected in the 7% separating gel. Four different polyacrylamide isozyme patterns were exhibited by the three species of mycobacteria (Fig. 2 and 3). Two of the four patterns were attributed to the species *M. chelonae*. All of the *M. chelonae* strains tested possessed two sulfatase bands, C_1 and C_3 . A third band, C_2 , with electrophoretic mobility intermediate between C_1 and C_3 , was seen in two of the three strains of *M. chelonae* subsp. *chelonae*. However, the type strain of *M. chelonae*, which is classified in the subspecies *chelonae*, lacked the C_2 band. The *M. fortuitum* zymogram consisted of four bands. The most anodal band (F_1) was well isolated. A second zone consisting of a diffusely stained area was further resolved by increasing the length of the electrophoretic run to reveal three closely migrating bands (F_2 , F_3 , F_4). The *M. peregrinum* zymogram also consisted of four bands. The more anodal bands (P_1 , P_2) had electrophoretic mobilities similar to the mobility of the F_1 band of *M. fortuitum*. Of the more cathodal bands (P_3 , P_4), the P_3 band had a mobility similar to the *M. fortuitum* bands F_2 , F_3 , and F_4 of the whole extract.

Fractions of *M. fortuitum* extract containing sulfatase activity originally separated by agarose electrophoresis were then eluted and run on polyacrylamide. The fast fraction (S_4), when eluted from agarose, produced in polyacrylamide a single band corresponding to the F_2 band of the whole extract. The slow band (S_3) from agarose produced 3 bands in polyacrylamide corresponding to the bands F_2 , F_3 , F_4 of the whole extract.

Neither of the agarose electrophoresis fractions produced a band having an F_1 mobility in polyacrylamide electrophoresis. The F_1 band seen in polyacrylamide electrophoresis of the unfractionated extract was much less intensely stained than the F_2 , F_3 , and F_4 bands. It is possible that it was inactivated or diluted beyond detection during the elution of the fractions from the agarose.

Immunological characterization. One arc with sulfatase activity was detected when extracts of *M. fortuitum* and *M. peregrinum* reacted with their homologous antisera in immunodiffusion and immunoelectrophoresis. Both of these extracts, when reacted with the heterologous antisera, also produced single arcs. None of the extracts from *M. chelonae* strains exhibited arcs with sulfatase activity when either *M. fortuitum* or *M. peregrinum* antisera were employed; nor did these extracts cause a deflection of the precipitin line resulting from homologous reactions in immunodiffusion. Immunoelectrophoresis of the *M. fortuitum* fractions S_3 and S_4 against antiserum to *M. fortuitum* was performed to determine which band produced the arc with sulfatase activity. The S_4 fraction failed to react with the antiserum, whereas the S_3 fraction produced one arc with sulfatase activity having the same electrophoretic mobility as the sulfatase arc seen with the whole extract.

Antisera to *M. fortuitum* absorbed with an excess of *M. peregrinum* extract failed to react in immunodiffusion experiments with *M. fortuitum* sulfatase. *M. peregrinum* antisera absorbed

with an excess of *M. fortuitum* extract also failed to react when tested with *M. peregrinum* sulfatase. Absorption of either antiserum with *M. chelonae* did not inhibit the reaction of the sulfatase in extracts from the homologous species.

DISCUSSION

The present study has shown that electrophoretic analysis of arylsulfatase isozymes affords a method for distinguishing the three species in the *M. fortuitum* complex from each other.

Electrophoresis in agarose followed by staining techniques specific for sulfatase produced isozyme patterns which were consistent for *M. fortuitum*, *M. peregrinum*, and *M. chelonae*, independent of the concentration of the extracts or the age of the culture from which the extract was prepared. However, polyacrylamide gel electrophoresis separated the sulfatases according to charge and molecular size and produced more complex zymograms than those from agarose electrophoresis. Increasing the length of time of electrophoresis in polyacrylamide was necessary to separate some of the closely migrating bands of *M. fortuitum*.

Agarose electrophoresis produced one zymogram pattern for all strains of *M. chelonae*. However, polyacrylamide electrophoresis of the four strains of *M. chelonae* produced two different sulfatase patterns. These two zymograms, obtained with strains currently classified in the same subspecies, *M. chelonae* subsp. *chelonae*, indicate that some variation exists within this subspecies.

Immunological characterization correlates well with agarose and polyacrylamide electrophoresis results. *M. fortuitum* and *M. peregrinum* had zymograms that were similar but distinguishable, and immunological assays showed cross-reactions of *M. fortuitum* and *M. peregrinum* sulfatases. *M. chelonae* had signifi-

FIG. 2. Sulfatase isozymes from polyacrylamide gel electrophoresis.

FIG. 3. Schematic drawing of sulfatase isozymes from polyacrylamide gel electrophoresis.

cantly different electrophoretic bands of sulfatase activity from *M. fortuitum* and *M. peregrinum* and did not cross-react with anti-*fortuitum* or anti-*peregrinum*.

Absorption of the antiserum against *M. fortuitum* with extracts of *M. peregrinum* and of the antiserum against *M. peregrinum* with extracts of *M. fortuitum* removed precipitating antibodies to the sulfatases of the homologous species. It was shown that the slowly migrating band (S₃) of *M. fortuitum* extract which had been eluted after agarose electrophoresis reacted with antiserum against *M. fortuitum* in immunoelectrophoresis to produce a single arc with sulfatase activity. It can be presumed that the slow band (S₂) of *M. peregrinum* extract also is responsible for its single arc in immunoelectrophoresis. The slow band of *M. chelonae* (S₁), whose migration is significantly different from the slow band of *M. fortuitum* or *M. peregrinum*, is apparently incapable of reacting with antisera against *M. fortuitum* or *M. peregrinum*, or at least not in quantities detectable by the staining method employed. It was possible that the absence of a visible reaction between the antisera and the *M. chelonae* extract was due to an immune complex formation in which the functional portion of the enzyme was sterically hindered. However, absorption of antisera against *M. fortuitum* or *M. peregrinum* with *M. chelonae* extract did not remove precipitating antibodies to the sulfatases of *M. fortuitum* (S₃) or *M. peregrinum* (S₂). In addition, immunodiffusion of extracts of *M. fortuitum* or *M. peregrinum* with their respective antisera resulted in a sulfatase arc which was not deflected or diminished when an extract of *M. chelonae* was placed in an adjacent well. This would indicate that the sulfatases of *M. chelonae* did not share antigenic constituents with S₃ of *M. fortuitum* or S₂ of *M. peregrinum*.

We cannot exclude the possibility that the antisera contained antibodies that might have inactivated the S₁, S₅, or S₄ sulfatases. However, our inclination has been to consider the absence of demonstrable antibodies to these sulfatases (S₁, S₅, and S₄) as being due to the poor humoral response of the animals rather than to the inactivation of the sulfatases by antibody combining with the functional moiety of the protein. In our studies with other enzymes, we have found that antibodies were elicited more easily in animals when a partially purified or an enriched enzyme fraction was used for immunization rather than by injecting an unfractionated extract. Since this was the initial study of the mycobacterial sulfatases, we chose first the use of unfractionated extracts for immunization. However, in pursuing

our investigations, we plan to use isolated enzyme fractions of the extract for immunization and characterization of the sulfatases.

This study has demonstrated the existence of unique sulfatase zymograms for *M. fortuitum*, *M. peregrinum*, and *M. chelonae*. Although one of the sulfatase isozymes of *M. fortuitum*, and *M. peregrinum* showed immunological identity, the difference in electrophoretic mobility provided recognizable patterns which served to distinguish the two species. These results support the comment in *Bergey's Manual*, 8th ed. (14), that *M. peregrinum* might be a synonym for *M. fortuitum* and the suggestion of Kubica (9) that these organisms be placed in one species and distinguished by subspecies designation. *M. chelonae* sulfatases shared no immunological identity with *M. fortuitum* or *M. peregrinum* and had distinctly different sulfatase zymograms.

The single pattern from agarose and the similarity of the two patterns from polyacrylamide gel for *M. chelonae* would support previous studies (7, 8, 17) indicating that *M. chelonae*, *M. abscessus*, and *M. borstelense* be classified in the same species as that of *M. chelonae*, with subspecies designations. However, the polyacrylamide zymograms do not correspond to the two current subspecies designations, but show a variation in the sulfatases of the subspecies *chelonae*.

The staining technique employed in this study provides a simple method for analyzing mycobacterial extracts with antisera directed against a variety of antigens while obtaining results specific for the enzymes being studied. These procedures employing different enzyme substrates could be applied to the characterization of other organisms whose laboratory identification or taxonomic status is questionable.

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