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Characterization of 2,3-Dihydroxybenzoic Acid from *Nocardia asteroides* GUH-2

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Culture filtrates of virulent *Nocardia asteroides* GUH-2 after growth in acetate minimal medium displayed an absorbance maximum at 320 nm. After isolation by polyamide extraction and anion chromatography, a UV-active compound with this absorbance was shown to be 2,3-dihydroxybenzoic acid (DHB) by nuclear magnetic resonance, gas chromatographic, and mass spectrometric techniques. DHB production under several culture conditions was quantified by a standard high-pressure liquid chromatography assay. Under iron deficiency conditions, *N. asteroides* GUH-2 excreted up to 11 mg of DHB per liter into the culture medium. No DHB was detected when *N. asteroides* GUH-2 was grown in an iron-rich medium. With the less virulent strain *N. asteroides* 10905, DHB was not found under any condition tested.

Nocardiae are filamentous soil bacteria, some of which have the ability to evade the bactericidal killing by human phagocytes and thus give rise to infections in humans (3, 31). The mechanisms of pathogenesis are complex (2, 5, 13) and not fully understood. However, they appear to be associated, in part, with an active metabolism and possibly the excretion of metabolites since, for example, log-phase cells of *Nocardia asteroides* GUH-2 are more virulent and grow better within macrophages than do stationary-phase cells of the same culture (14).

Secondary metabolites (25) are involved in the virulence mechanisms of many bacterial pathogens, especially those causing disease in plants (12, 15), but so far, they have not attracted much attention as potential virulence factors for pathogens causing human disease (32), microbial iron chelators being an exception (26, 34). *Nocardia* sp. strain SC11340 produces iron-chelating agents, so called siderochelins (24), which are chemically related to ferrorosamine A from the plant pathogen *Erwinia rhapontici* (17). Also, L-2,5-dihydrophenylalanine (DHP), a toxic secondary metabolite excreted by the fireblight pathogen *Erwinia amylovora* (16), is a common metabolic product of actinomycetes (25). These observations suggested to us that the secondary metabolism of *N. asteroides* is important for its pathogenicity. The aim of the present study, therefore, was to investigate whether low-molecular-weight metabolites excreted by the virulent strain *N. asteroides* GUH-2 would differ from those excreted by the less virulent strain *N. asteroides* 10905.

(An abstract of this work was presented at the 6th International Symposium on the Biology of Actinomycetes, Debrecen, Hungary, 1985.)

MATERIALS AND METHODS

Organisms and growth conditions. *N. asteroides* GUH-2 was isolated from a human with a fatal infection at Georgetown University Hospital, Washington, D.C., and *N. asteroides* 10905 was supplied by J. Rozanis at the University of Western Ontario, London, Ontario, Canada. The virulence of these strains has been subject to earlier investigations (for example, see references 2, 5, and 13). Except for initial studies, growth was achieved on a minimal medium containing the following (grams per liter): $(NH_4)_2HPO_4$, 1; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; and glutamic acid, 10, or sodium acetate, 10. The media were inoculated with 1 drop (0.05 ml) of a 5-day-old brain heart infusion culture of the particular organism per 50 ml of minimal medium. The cultures were incubated for 10 days on a rotary shaker at 37°C (1).

Isolation of DHB. Typically, bacteria were removed by centrifugation at 9,000 × *g* for 10 min, and the medium was filtered through Nalgene LSCN 500-ml filter units or Nuclepore Membra-fil 0.45-μm-pore-size filters. Upon acidification of the medium to pH 2.7 with sulfuric acid, CO_2 developed (degassing was facilitated by stirring) and a precipitate formed which was removed by filtration through Whatman 2^V filter paper. Combined culture filtrate (8 liters) was then passed over a polyamide column (16 by 2.5 cm; Macherey Nagel Co., Düren, Federal Republic of Germany), and the column was washed with H_2O (300 ml) and eluted with 10% aqueous NH_3. The eluate (100 ml) was evaporated in vacuo, and the residue was dissolved in 5 ml of H_2O. To assist solubility, gaseous NH_3 was blown into the flask. The solution thus obtained was applied to a DEAE-Sephadex A25 column (14 by 2.5 cm) and chromatographed with a linear gradient of triethylammonium bicarbonate (TEAB) generated from 0.01 and 0.3 M TEAB (500 ml each), followed by 1 liter of 0.3 M TEAB. The effluent was passed through a flowthrough cell (10-mm path length), and the UV A_{320} was monitored with a Beckman DB spectrophotometer. Two major peaks were observed (see Fig. 2). From the first peak, 2,3-dihydrobenzoic acid (DHB) was collected.

Test for ferrorosamines-siderochelins and DHP. *N. asteroides* was grown for 13 days in 1 liter of Middlebrook 7H9 medium (plus 0.5% glucose; Difco Supplementary Literature (Code 0713), p. 244 1968; Difco Laboratories, Detroit, Mich.) from which the ferric ammonium citrate had been omitted. Subsequent addition of ferrous sulfate to the culture filtrate did not result in the formation of a pink pigment, which indicates the probable absence of ferrorosamines (17). To test for DHP, the culture filtrate (acetate minimal me-

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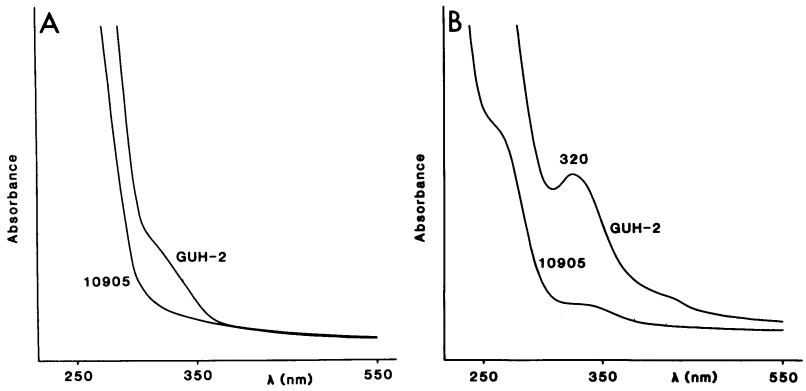

FIG. 1. Absorbance spectra of culture filtrates from *N. asteroides* GUH-2 and *N. asteroides* 10905 after growth in glutamic acid minimal medium (A) or acetate minimal medium (B).

dium), after being passed through a polyamide column (see above), was extracted further with a Dowex 50WX8 column (22 by 2.5 cm, H^+ form), and the column was washed with 1 liter of deionized water and eluted with 1 liter of 10% aqueous NH_3. The eluate was evaporated in vacuo at 30°C to give 1.2 g of a hygroscopic residue. This residue was subjected to reversed-phase thin-layer chromatographic analysis on HETLC-RPS-F (scored to 5 by 5 cm; Analtech, Newark, Del.) using 5% aqueous methanol as the developing solvent. Authentic DHP from *E. amylovora* (G. Feistner, manuscript in preparation) was included as a reference (R_f = 0.5; brick-orange color reaction with ninhydrin).

Methyl derivatives of DHB. Diazomethane, generated by standard procedures from 1 g of Diazald (Aldrichim. Acta **16**:3, 1983), was percolated through a solution of 1 mg of DHB (from either *N. asteroides* or ICN-K & K Laboratories, Inc., Irvine, Calif.) in 10 ml of methanol. The sample was concentrated to 1 ml, and 1 μl was injected for gas chromatographic (GC) analysis (2 to 3 μl for GC-mass spectrometric [MS] analysis).

High-pressure liquid chromatography of nocardial culture filtrate. A general procedure for the high-pressure liquid chromatographic (HPLC) separation of phenolic compounds (33) was used with the following equipment: Spectra Physics model 3500B pump and injector, Bio-Rad ODS-10 column (250 by 4 mm) and ODS-5 guard column, and Spectra Physics SP 8400 UV/VIS detector. The injection of 500 μl of nocardial culture filtrate followed by absorbance recording at 320 nm (detection range, 0.01 absorbance units full scale) resulted in the unambiguous detection of 100 μg of DHB per liter of culture filtrate. Samples were run in duplicate.

Instrumental analysis. UV absorbance spectra were determined with an Aminco DW-2[a] UV/VIS spectrophotometer (Travenol Laboratories, Inc., Round Lake, Ill.). GCs were recorded with a Hewlett-Packard (Palo Alto, Calif.) 5880A GC and a 12.5-m (0.2-mm inner diameter) Hewlett-Packard cross-linked dimethyl silicone capillary column. Mass spectra were obtained with a Finnigan MAT model 3200 MS operated in the electron impact (EI) ionization mode (70 eV ionization energy). Underivatized DHB samples were introduced via direct insertion, whereas the methyl derivatives were first chromatographed on a DB-1 (30 m, 0.25-μm film thickness) fused silica capillary column (J & W Scientific, Inc., Rancho Cordova, Calif.) housed in a Finnigan 9500 GC. Partial identification was possible by comparison with the National Bureau of Standards library of mass spectra (38,762 entries). High-resolution MS ($m/\Delta m$ = 10,000, 10% valley definition) and chemical ionization MS (2×10^{-5} millibar methane) were achieved with a VG ZAB-HS instrument (VG Analytical, Manchester, United Kingdom) coupled to a VG 11/250 data system. For nuclear magnetic resonance (NMR) measurements, nocardial DHB (5 mg) was dissolved in ca. 1 ml of D_2O (with the aid of a small amount of gaseous NH_3), and the spectra were recorded with a 500 MHz Fourier transform NMR spectrometer (Nicolet Instrument Corp., Madison, Wis.); chemical shifts are given relative to the solvent peak (DHO 4.75 ppm).

Reference numbers. Chemical Abstracts Service reference numbers are as follows: DHB, 303-38-8; 2,3-dimethoxybenzoic acid methyl ester, 2150-42-7; proferrorosamine A, 26927-08-2; DHP, 16055-12-2; siderochelins, 77550-88-0.

RESULTS

Excretion of iron chelators of the ferrorosamine type can easily be detected by growing the organism in an iron-deficient medium and by subsequently adding $FeSO_4$ to the culture filtrate: a pink ferrous iron complex forms (17). This was not observed with either *N. asteroides* GUH-2 or *N. asteroides* 10905 after growth in an iron-deficient medium. Furthermore, the culture filtrate of *N. asteroides* GUH-2 did not appear to contain DHP. However, the spent culture broth of *N. asteroides* GUH-2 displayed a UV absorbance maximum at 320 nm which was significantly more intense than that for *N. asteroides* 10905 (Fig. 1). Therefore, efforts were concentrated in identifying the compounds responsible for this A_{320}. This material could be adsorbed on polyamide, indicating a possible catechol-type compound. Since it was also retained by an anion-exchange column, it was tentatively considered to be a phenolic acid. When the polyamide extract was chromatographed on DEAE-Sephadex A25, two major peaks were observed (Fig. 2). Material collected from the first peak gave the ^1H NMR spectrum shown in Fig. 3. The coupling patterns and the absolute magnitude of the coupling constants (7.30 ppm, d, 7 Hz, 1H; 6.98 ppm, d, 8.5 Hz, 1H; 6.77 ppm, t, 8.0 Hz, 1H) seen in the aromatic region suggested three neighboring protons. This was corroborated by double-resonance irradiation at the frequency of the triplet, which caused both doublets to collapse to singlets. The ^1H NMR spectrum also showed signals for triethylammonium ions (derived from the chromatography buffer): $-CH_2-$ (10H) and $-CH_3$ (1.3 ppm, t, 15H). Since one triethylammonium ion contains nine methyl and six methyl-

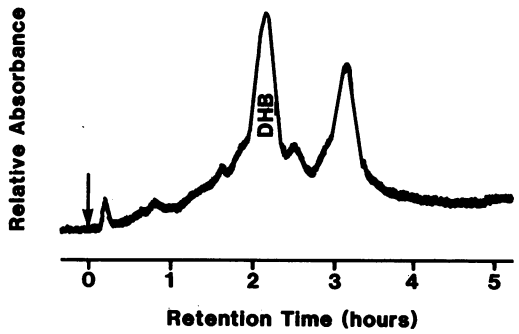

FIG. 2. Separation of the polyamide extract from GUH-2 culture filtrate on DEAE-Sephadex A25 (25 by 140 mm), using first a linear gradient of 0.01 to 0.3 M TEAB (500 ml each) and then 1 liter of 0.3 M TEAB isocratically.

ene protons, the relationship toward the aromatic protons was not stoichiometric. However, the signal for the methylene protons was split into three: 3.23 ppm, q, 2H; 3.14 ppm, q, 6H; 3.03 ppm, q, 2H. This was indicative of one stable triethylammonium salt function and two others that were decomposed in vacuo before the sample preparation for NMR spectroscopy. The latter result is typical for phenols.

EI-MS revealed a molecular weight of 154, and high-resolution MS suggested the elemental composition $C_7H_6O_4$ (chemical ionization MS: $[M + H]^+$, found 155.0341, calculated 155.0344). Three acidic protons were confirmed by D_2O exchange, which shifted the molecular ion to m/z 157. A library search suggested a dihydroxybenzoic acid but it did not permit ambiguous assignment of which isomer was present. This information, however, could be deduced from the NMR spectrum, which, in addition to three neighboring protons, suggested an asymmetrical molecule, thus leaving DHB as the most likely structure.

The structure assignment was further corroborated by GC of the methylation products from authentic DHB and from DHB of nocardial origin. In both cases (Fig. 4), compounds with virtually the same retention times were obtained, although the absolute intensities for the two batches differed. Cochromatography of the two batches also resulted in only four peaks. Obviously, the methylation did not proceed completely to the trimethylated product, but also gave the two isomeric monohydroxy-monomethoxy-benzoic acid methyl esters and a small amount of another derivative, which was not identified, but which most likely was the dihydroxybenzoic acid methyl ester. To assign the different peaks, GC was coupled with MS. Thus, it could be shown that the compound with the longest retention time (Fig. 5) was 2,3-dimethoxybenzoic acid methyl ester. Other than for the parent compound (DHB), the trimethylated derivative (2,3-dimethoxybenzoic acid methyl ester) permitted a clear distinction from other positional isomers (20) and thereby confirmed the conclusions of DHB.

Methylation of DHB also gave rise to 3-hydroxy-2-methoxybenzoic acid methyl ester and 2-hydroxy-3-methoxybenzoic acid methyl ester. No mass spectra of these

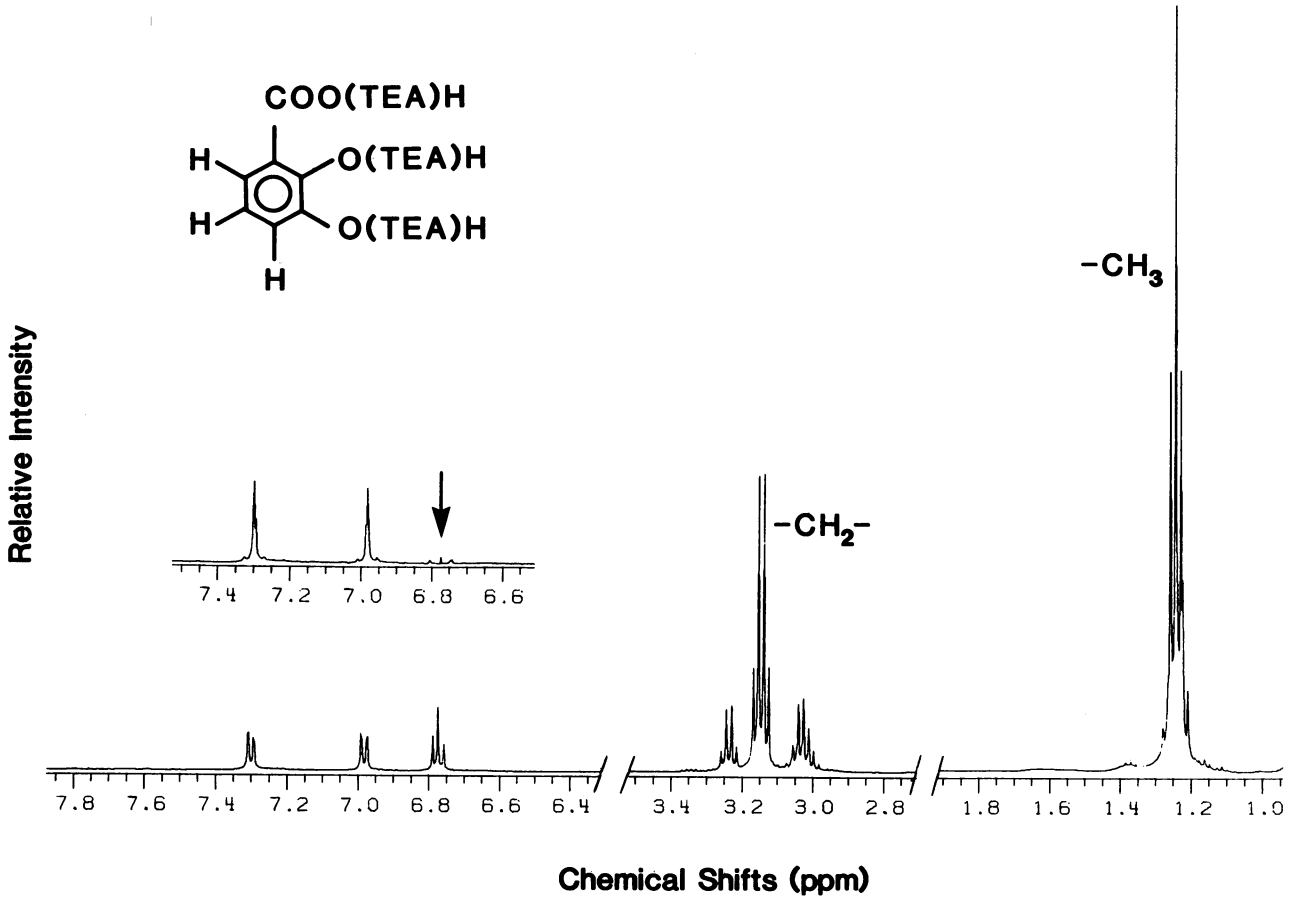

FIG. 3. ^1H NMR spectrum (500 MHz) of nocardial DHB isolated in its TEA form after separation in a TEAB buffer (Fig. 2).

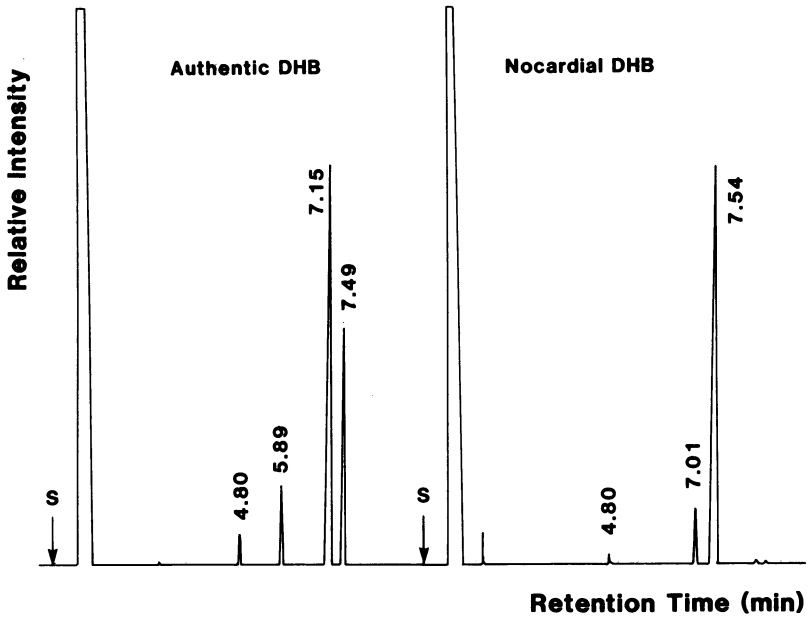

FIG. 4. GC separation (1 min at 100°C, 15°C/min to 135°C, 1°C/min to 150°C) of the reaction product of DHB (both synthetic and of nocardial origin) with diazomethane, using a flame ionization detector. s, Start of chromatogram.

authentic compounds were available to us, but the two isomers could be distinguished on grounds of the different *ortho* effects displayed by them. 3-Hydroxy-2-methoxybenzoic acid methyl ester, like DHB, showed predominantly the loss of a methoxy radical (Fig. 6) (31 mass units) from the ester function, whereas 2-hydroxy-3-methoxybenzoic acid methyl ester with a free hydroxyl group in the *ortho* position lost predominantly methanol (32 mass units). This latter compound, on the other hand, with no hydrogen atom *ortho* to the phenol group, did not expel H_2O (18 mass units), whereas 3-hydroxy-2-methoxybenzoic acid methyl ester did.

For the same methylation mixture, the peak intensities for the individual compounds detected in the GC with the flame ionization detector were different from those obtained with the MS detector. Specifically, the less methylated compounds gave a relatively stronger signal in the flame ionization detector mode (Fig. 4) than in the integrated ion count mode of the MS (Fig. 5). Since phenols have an inherent tendency to form anions rather than cations, this may be due to less efficient positive ionization of free phenols as compared with phenyl methyl ethers under EI-MS conditions.

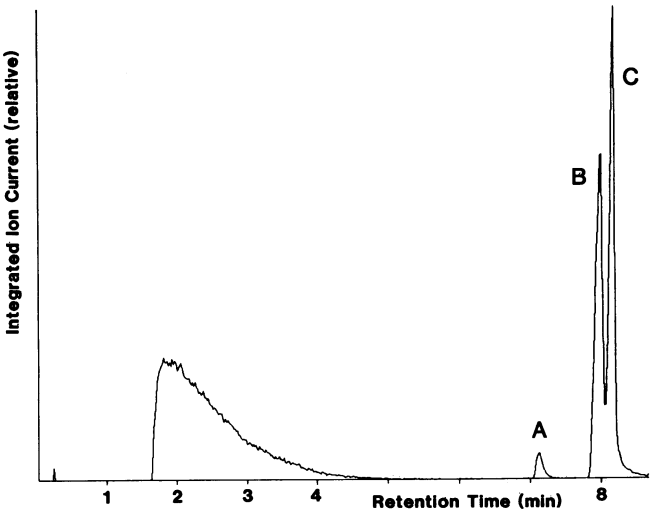

FIG. 5. Acquisition total ion current trace of a GC-MS run (10°C/min from 100°C) of methylated authentic DHB (same batch as in Fig. 4). A, B, and C correspond to panels d, c, and b, respectively, in Fig. 6.

Because of the extra manipulations required and the multiplicity of peaks, GC-MS was not the method of choice to quantitate differences in the production of DHB by either *N. asteroides* GUH-2 or *N. asteroides* 10905. Since UV absorbance of this compound at 320 nm allowed direct, easy measurements, a general HPLC method for phenols (33) was utilized. Thus, the UV-active samples with the same retention time as authentic DHB were collected and verified by EI-MS. Quantification through peak height measurement yielded the following results (figures refer to average of duplicate experiments): (i) *N. asteroides* GUH-2 produced 2.7 ± 1.0 mg of DHB per liter in acetate minimal medium (ca. 10 μg of DHB per mg of GUH-2 [wet weight]); (ii) in glutamic acid minimal medium, which supported the growth of *N. asteroides* GUH-2 better than did the acetate minimal medium, production of DHB increased to 11.2 ± 1.3 mg/liter (ca. 30 μg/mg [wet weight]); (iii) addition of 40 mg of ferric citrate per liter (1.6 nM) totally suppressed the production of DHB, whereas 1.6 nM of ammonium citrate did not; (iv) *N. asteroides* 10905, even under iron deficiency conditions, did not produce DHB (Fig. 7); (v) the HPLC revealed that A_{320} was due to several nocardial metabolites rather than DHB alone. These additional compounds will be subjected to further analysis.

DISCUSSION

Dihydroxybenzoic acid is a secondary metabolite that is found with a variety of bacteria (9, 10, 22, 23, 25), including *Nocardia opaca* (27). This is the first report of DHB being

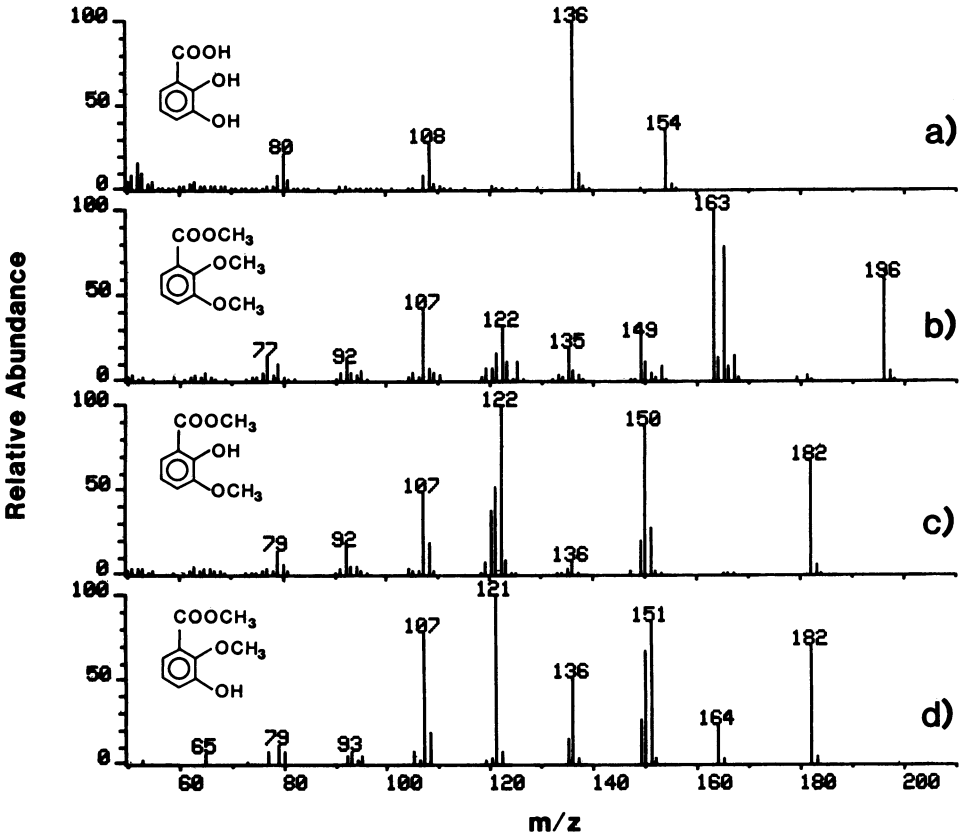

FIG. 6. EI mass spectra of DHB (a), 2,3-dimethoxy benzoic acid methyl ester (b), 2-hydroxy-3-methoxybenzoic acid methyl ester (c), and 3-hydroxy-2-methoxybenzoic acid methyl ester (d); panels b through d correspond to A through C in Fig. 5.

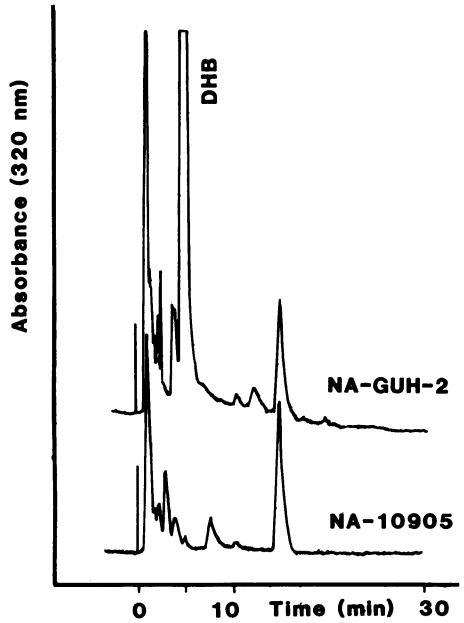

FIG. 7. Reversed-phase (C_{18}) HPLC of 500 μl of culture fitrate of *N. asteroides* GUH-2 and *N. asteroides* 10905 after growth on glutamic acid minimal medium for 68 hours; here the peak for DHB is overloaded to emphasize the difference between the two nocardial strains. The chromatographic conditions were as follows: flow, 3.2 ml/min, solvent, water-methanol-formic acid (80:15:5, vol/vol/vol), and absorbance measurements at 320 nm, 0.01 absorbance units full scale. The wet weights observed in this particular example were ca. 400 mg/50 ml of culture for strain GUH-2 and ca. 65 mg/50 ml of culture for strain 10905; however, in a separate experiment in which a wet weight of ca. 200 mg/50 ml of culture was recorded for strain 10905 (growth for 82 hours), the respective culture filtrate gave almost the same chromatogram as the one shown here; especially, no DHB could be seen either.

produced by pathogenic strains of *Nocardia* species. Since virulent *N. asteroides* GUH-2 produces large amounts of DHB and the less virulent *N. asteroides* 10905 excretes no detectable DHB, it is tempting to speculate that DHB is important for the virulence and the intracellular survival of *N. asteroides* in host phagocytes. Both *N. asteroides* GUH-2 and *N. asteroides* 10905 are able to grow under iron deficiency conditions, but as the faster-growing organism of the two, *N. asteroides* GUH-2 seems to be better able to cope with the iron deficit. This may be due to the ability of *N. asteroides* GUH-2 to respond to iron starvation with the excretion of large amounts of the siderophore DHB, a mechanism which apparently is not available to *N. asteroides* 10905. Since DHB is able to remove iron from human plasma (19, 21), DHB may indeed enable *N. asteroides* GUH-2 to survive in an iron-deficient environment such as a human host. In addition, *N. asteroides* GUH-2 may benefit from the production of DHB by taking advantage of DHB being a radical scavenger (7, 8, 11, 28, 29), capable of neutralizing bactericidal oxygen radicals (4) which are produced during phagocytosis (6, 30). We think that this characteristic of DHB may explain, in part, the known extraordinary resistance of *N. asteroides* toward

oxidative killing mechanisms by mammalian phagocytes (2, 18), in particular those experimental results which cannot be accounted for by superoxide dismutase and catalase (18). With regard to the two *Nocardia* strains used in this study, DHB may partly explain the observation made earlier in this laboratory (13) that with equivalent inocula, *N. asteroides* GUH-2 largely survives phagocytosis by macrophages, whereas *N. asteroides* 10905 is much less able to do so. Preliminary observations on DHB production in several additional strains have confirmed that some strains of *N. asteroides* produce DHB while other strains do not (data not included). So far, in every instance studied, the organisms that produce DHB are more virulent for mice than those that do not produce DHB. The study of mutants of virulent *N. asteroides* which have lost the ability to produce DHB is the logical next step.

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