The polar lipids and free sugars of *Frankia* in culture

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To further the identification of characteristics common to *Frankia* strains, the phospholipids and sugars which are readily extracted with chloroform–methanol from whole cells were examined using nine strains of *Frankia*. Separation of extract components was achieved by thin-layer chromatography. In agreement with previous reports we have identified two phospholipids, phosphatidyl inositol and diphosphatidyl glycerol, in eight of the strains tested. All strains contained a glucose disaccharide which we have tentatively identified as trehalose on the basis of (*i*) comparative thin-layer and paper chromatography with standards, (*ii*) examination of the hydrolyzed product, and (*iii*) tests for reducing versus nonreducing sugars. In addition, a hexose with the chromatographic properties of glucose was observed in cultures grown on M6B complex medium. The presence of the phospholipids in *Frankia* strains was not affected by the age of the culture or the media on which they grew. However, the disaccharide was not present in cultures over 60 days of age, suggesting it is involved in metabolic processes. In addition, comparisons were made between extracts from *Frankia* and the nitrogen-fixing cyanobacterium *Anabaena variabilis* to determine if the glycolipids which are unique to *Anabaena* heterocysts are present in *Frankia*. These two organisms appear to have an unidentified lipid in common, but glycolipids comparable with those in *Anabaena* were not found in the *Frankia* cultures examined.

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Dans le but de poursuivre l'identification des caractéristiques communes des souches de *Frankia*, nous avons étudié, chez neuf souches de *Frankia*, les phospholipides et les sucres qui peuvent être facilement extraits au chloroforme–méthanol à partir des cellules entières. Les composants ont été séparés des extraits par chromatographie sur couche mince. En accord avec les données publiées antérieurement, nous avons identifié deux phospholipides chez huit des neuf souches: le phosphatidyl inositol et le diphosphatidyl glycérol. Toutes les souches contiennent un disaccharide de glucose, que nous avons expérimentalement identifié comme du tréhalose en nous fondant sur (*i*) son comportement chromatographique sur couche mince et sur papier par comparaison avec des étalons, (*ii*) la nature de ses produits d'hydrolyse et (*iii*) des essais pour les sucres réducteurs ou non-réducteurs. De plus, un hexose ayant les propriétés chromatographiques du glucose a été observé dans les cultures sur le milieu complexe M6B. La présence des phospholipides dans les souches de *Frankia* n'est pas affectée par l'âge des cultures, ni par le milieu sur lequel les souches croissent. Cependant, le disaccharide ne se rencontre pas dans les cultures âgées de plus de 60 jours, ce qui laisse croire qu'il est utilisé dans les processus métaboliques. En outre, nous avons comparé les extraits de *Frankia* à ceux d'une cyanobactérie fixatrice d'azote, *Anabaena variabilis*, pour voir si les glycolipides uniques aux hétérocystes d'*Anabaena* se rencontrent chez *Frankia*. Ces deux organismes semblent posséder en commun un lipide non identifié, mais on ne retouve pas de glycolipides comparables à ceux d'*Anabaena* dans les cultures de *Frankia* qui ont été examinées.

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Introduction

The characterization of *Frankia* as a distinctive genus within the aerobic Actinomycetales is based on (*i*) its unique combination of morphological structures in culture (Newcomb *et al.* 1979); (*ii*) its cell wall chemistry (Lechevalier and Lechevalier 1979); and (*iii*) its capacity to initiate nitrogen-fixing root nodules in a wide range of woody dicotyledonous plants (Torrey 1979). Although many distinct strains within the genus *Frankia* are recognized, relationships among them are not yet clear. Baker (1982) provided a comprehensive list of the characteristics of many strains which includes pigmentation, cell wall type, whole-cell sugar pattern, phospholipid type, serogroup, infective and effective capacity, and host compatibility.

Lipid composition has been used by several workers as a taxonomic criterion in the study of actinomycetes. Mordarska and Mordarski (1970) used thin-layer chromatography of lipid extracts to differentiate among strains of *Nocardia*, *Corynebacterium*, and *Mycobacterium*. Lechevalier *et al.* (1971) used long-chain mycolic acids to distinguish between strains of these

same genera. In a more recent report, Lechevalier *et al.* (1981) considered differences in the phospholipid composition of motile actinomycetes, in conjunction with other criteria, to suggest that motility arose as a consequence of parallel evolution. The phospholipid content of *Frankia* has been categorized by Lechevalier *et al.* (1982) as phospholipid type I, consisting of phosphatidyl inositol (PI), phosphatidyl inositol mannosides (PIM), diphosphatidyl glycerol (DPG), and lyso-diphosphatidyl glycerol (lysoDPG) (M. P. Lechevalier, personal communication).

Sugar analyses have also been used in taxonomic studies of actinomycetes. Lechevalier *et al.* (1979) reported that whole-cell sugar hydrolysates, in conjunction with cell wall type and morphological characteristics, provided a useful criterion for the taxonomic placement of actinomycetes. Lechevalier and Lechevalier (1979) examined the whole-cell sugar pattern of several *Frankia* strains and found variation among strains in this characteristic. Most strains are whole-cell sugar-type D with the exception of isolates from *Casuarina* (D11, G2), type B, and *Elaeagnus* (EuI1, EuI5) and *Purshia* (PtI1) which are unclassified.

The interest in *Frankia* lipids extends beyond taxonomy. The effect of extracted root lipids on the growth of *Frankia* has been investigated by Quispel and Burggraaf (1981), who reported that lipids have a stimulative effect on the growth of the endophytes of *Alnus viridis* and *Alnus glutinosa* in culture.

Torrey and Callaham (1982) hypothesized that lipids may be involved in excluding oxygen from the interior of *Frankia* vesicles. These vesicles possess specialized envelopes, which are birefringent in polarized light and are multilaminate, becoming visible when prepared by freeze fracture methods for electron microscopy. There is strong circumstantial evidence that *Frankia* vesicles are the site of dinitrogen fixation (Tjepkema *et al.* 1981) and that in a number of characters they may be analogous to the heterocysts of cyanobacteria such as *Anabaena*. In *Anabaena*, unique glycolipids have been identified as components of the inner laminated layer of the heterocyst wall (Nichols and Wood 1969; Winkenbach *et al.* 1972), a structure which is believed to serve as a protective barrier for the oxygen-sensitive enzyme nitrogenase within (Stewart 1973; Haury and Wolk 1978).

In this report we examine the phospholipid composition of nine *Frankia* strains and make preliminary characterizations of the sugars which are present in chloroform–methanol extracts from whole cells. To our knowledge, analyses of *Frankia* sugars have been performed exclusively after hydrolysis (Lechevalier and Lechevalier 1979); our extracts contain unhydrolyzed free sugars found in *Frankia*. In addition, we have made a comparison of *Frankia* polar lipids with those of

Anabaena variabilis to observe whether specific glycolipids comparable with those of heterocysts are found in *Frankia* lipid extracts.

Materials and methods

Bacterial and culture conditions

Frankia strains CpI1, ArI3, AgI1, CaI1, MpI1, EuI1, AvsI3, EAN1, and EUN1f were used for strain comparisons; most of the investigative work on lipid and sugar identification was done with CpI1 and ArI3. Cultures were grown either in stationary 125-mL Erlenmeyer flasks incubated at 25–28°C or in 10-L bottles with aeration and stirring at room temperature. No qualitative differences in lipid composition were observed among bacteria grown under these cultural conditions. However, all cultures used for strain comparison were grown under the stationary condition. Cultures for comparison were grown on M6B medium (modification of *Frankia* broth (Callaham *et al.* 1978)) or modified BAP medium (Murry *et al.* 1984) except for CaI1, which was grown on "S" medium of Lechevalier *et al.* (1983) supplemented with 10 g/L of glucose as carbon source, and EAN1 and EUN1f, which were grown on Qmod medium of Lalonde (Lalonde and Calvert 1979). BAP medium (Murry *et al.* 1984) was used for the aerated cultures in large bottles and was modified to BP medium by the omission of ammonium N for the induction of vesicles. Media carbon sources and concentration are given in Table 1. Culture ages varied from 7 to 180 days. *Anabaena variabilis* cultures were kindly supplied by Dr. M. Murry.

Extraction of lipids and sugars

Both lipids and sugars were extracted by modification of the Folch method (Folch *et al.* 1951). Cultures were harvested by centrifugation and washed once with distilled water. The resulting pellet was extracted with 20 volumes of chloroform–methanol 2:1 overnight under nitrogen at 3°C. The lower organic phase was removed with a pipet and saved. Twenty volumes of chloroform–methanol 1:2 were then added to the aqueous phase which contained cell debris and the resulting mixture was sonicated for 60 s to assure cellular disruption. This mixture was then extracted for 2 h at room temperature and then centrifuged to pellet the cell debris. The two extracts were then combined, evaporated to dryness under a stream of nitrogen, and redissolved in chloroform–methanol 2:1. Most

TABLE 1. Media and carbon sources

Medium (see text)	Carbon source	Concentration (mg/L)
BAP	Succinate	118
BP	Succinate	118
Modified BAP	Succinate	100
	Propionate	200
M6B	Yeast extract	5 000
	Glucose	10 000
Qmod	Yeast extract	500
	Glucose	10 000
S	Glucose	10 000

extraction mixtures contained 0.005% 2,6-di-*tert*-butyl-4-methylphenol (BHT) to prevent oxidation of lipids.

The method of Richtmeyer (1962) was used to extract sugars from *Frankia*. Cells were harvested by centrifugation and extracted with 95% ethanol for 30 min at room temperature. The cells were then washed in a Buchner funnel with 70% EtOH and the filtrate and extract combined, dried, and resuspended in 70% ethanol.

A Sephadex column was used to separate lipids from sugars and other nonlipids, as described by Wells and Dittmer (1963).

Identification of extract components

Extract components were separated by thin-layer chromatography (TLC) on silica-gel G plates (Fisher Scientific Co.) activated at 100°C for 10 min. Phospholipids were located with the molybdenum blue spray of Vaskovsky and Kostevsky (1968) and identified by cochromatography with standards (Sigma Chemical Co.). The solvent systems used for phospholipid separation were chloroform – methanol – distilled H_2O (60:35:5) and *n*-propanol – ammonium hydroxide – distilled H_2O (75:3:22) (Marks and Szulga 1965). Other TLC visualization sprays used were anisaldehyde (Stahl and Kaltenbach 1961) and diphenylamine (Dische 1929) for sugars and ninhydrin (Skipski and Barclay 1969) for amino groups. *Anabaena* extracts were separated using the solvent system chloroform – methanol – acetic acid – distilled H_2O (85:15:10:3.7) (Nichols and Wood 1968).

Sugars were identified by comparison with standards on both TLC plates and paper chromatographs using five solvent systems: butanol – pyridine – 0.1 N HCl (5:3:2); butanol – acetic acid – distilled H_2O (3:1:3); butanol – pyridine – distilled H_2O (6:4:3); propanol – ethyl acetate – distilled H_2O (7:1:2); and ethyl acetate – acetic acid – distilled H_2O (3:1:1) (Elbein 1967) (see Table 2). Sugars were visualized with silver nitrate (Trevelyan *et al.* 1950). Tests for reducing versus nonreducing sugars were done using the benzidine dip of Harris and MacWilliam (1954).

The hexose constituents of disaccharides were identified by acid hydrolysis and subsequent paper chromatography. Sugars were purified by preparative TLC and elution from the silica gel. The sugars were hydrolyzed with 3 N HCl at 100°C for 3 h. After hydrolysis, the acid was washed three times with petroleum ether to extract any hydrolyzed lipids that may be present. The petroleum ether extract was dried under nitrogen, resuspended in chloroform–methanol 2:1, and examined using TLC in a solvent system of hexane–ether 4:1 (Skipski and Barclay 1969). Visualization was with iodine vapor (Mangold and Malins 1960) and sulfuric acid – potassium dichromate spray (Privette and Blank 1962). After washing with petroleum ether, the remaining acid fraction was evaporated and the sugars were resuspended in distilled H_2O.

TABLE 2. Identification of the unknown hexose (*1*) and unknown disaccharide (*2*) on paper and thin-layer chromatograms

Compound	R_g*			R_f†	
	I	II	III	IV	V
Glucose	1.00	1.00	1.00	0.44	0.31
Trehalose	0.69	0.69	0.48	0.33	0.17
Unknown 1	0.95	1.00	1.05	0.44	0.30
Unknown 2	0.67	0.69	0.53	0.33	0.16

NOTE: Solvent systems are I, *n*-propanol – ethyl acetate – water, 7:1:2, descending paper; II, butanol – pyridine – 0.1 N hydrochloric acid, 5:3:2, descending paper; III, ethyl acetate – acetic acid – water, 3:1:1, descending paper; IV, *n*-propanol – ammonium hydroxide – water, 75:3:22, TLC; V, chloroform–methanol–water, 60:35:5, TLC.
*Ratio to glucose in solvent.
†Ratio to solvent front.

Results

Phospholipids

In agreement with Lechevalier's findings (Lechevalier *et al.* 1982) we have identified PI and DPG in *Frankia* lipid extracts (Fig. 1). These lipids reacted with molybdenum blue and general lipid-detection sprays, did not react with ninhydrin (as would nitrogenous phospholipids), and cochromatographed with PI and DPG standards.

In addition to PI and DPG, three faint spots which reacted with molybdenum blue were visible. These unidentified phospholipids travelled very close to PI and DPG and are probably the phosphatidyl inositol mannosides and lysodiphosphatidyl glycerol components reported by M. Lechevalier.

All *Frankia* strains, except EUN1f, clearly had both PI and DPG (Fig. 1). The EUN1f-strain extracts examined had large amounts of red pigment which obscured the phospholipids and made accurate identification impossible, even when a phosphate-specific spray was used. The other *Elaeagnus* isolates, EuI1 and EAN1, also had a reddish chloroform–methanol-soluble pigment which, however, did not interfere with phospholipid identification. No variation in phospholipid content was observed owing to changes in medium or age or morphology of culture.

FIG. 1. TLC of the total lipids of *Frankia* strains in the solvent system *n*-propanol – ammonium hydroxide – water, 75:3:22. Visualization: anisaldehyde – sulfuric acid spray. Fig. 1A. TLC plate. Fig. 1B. Tracing of TLC plate. *1*, AgI1; *2*, ArI3; *3*, AvsI3; *4*, CaI1; *5*, CpI1; *6*, PI standard; *7*, DPG standard; *8*, EAN1; *9*, EuI1; *10*, EUN1f; *11*, MpI1. DPG, diphosphatidyl glycerol; PI, phosphatidyl glycerol; UPL, unidentified phospholipid (see text); GLU, glucose; TRE, trehalose.
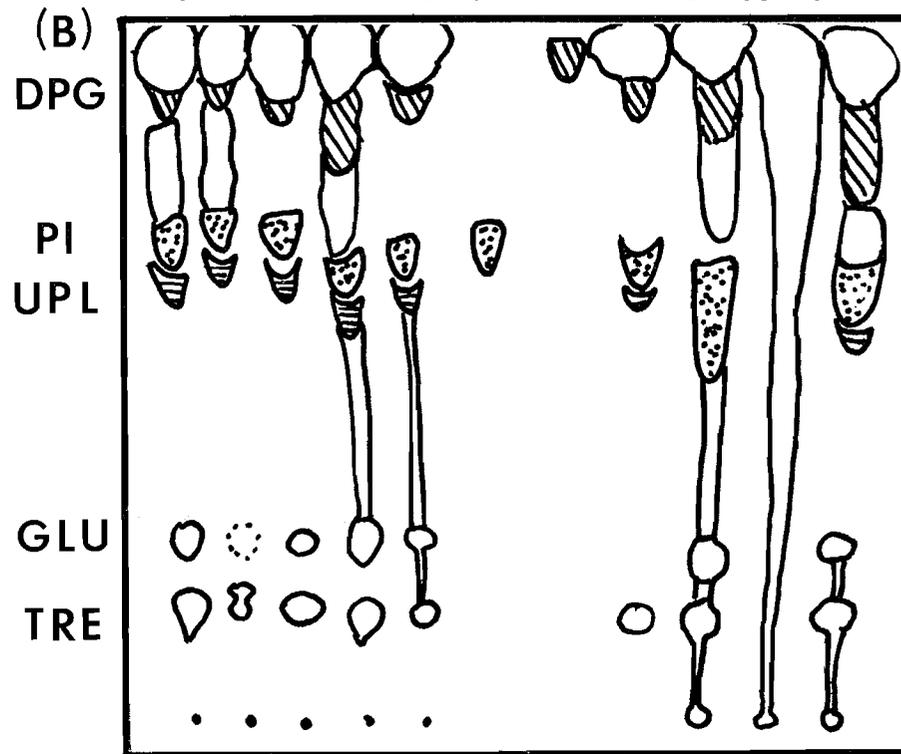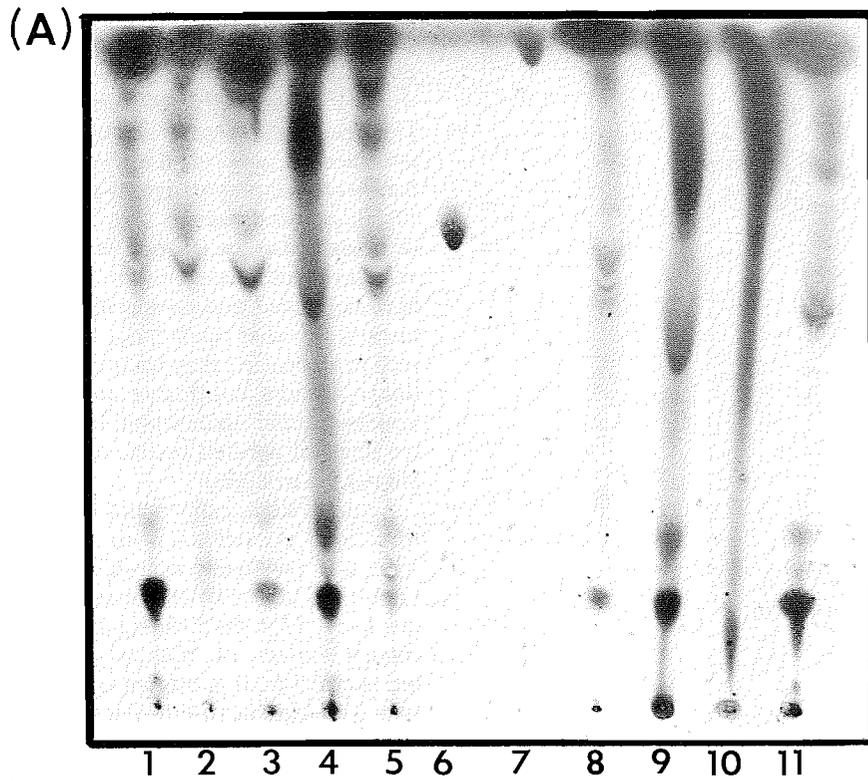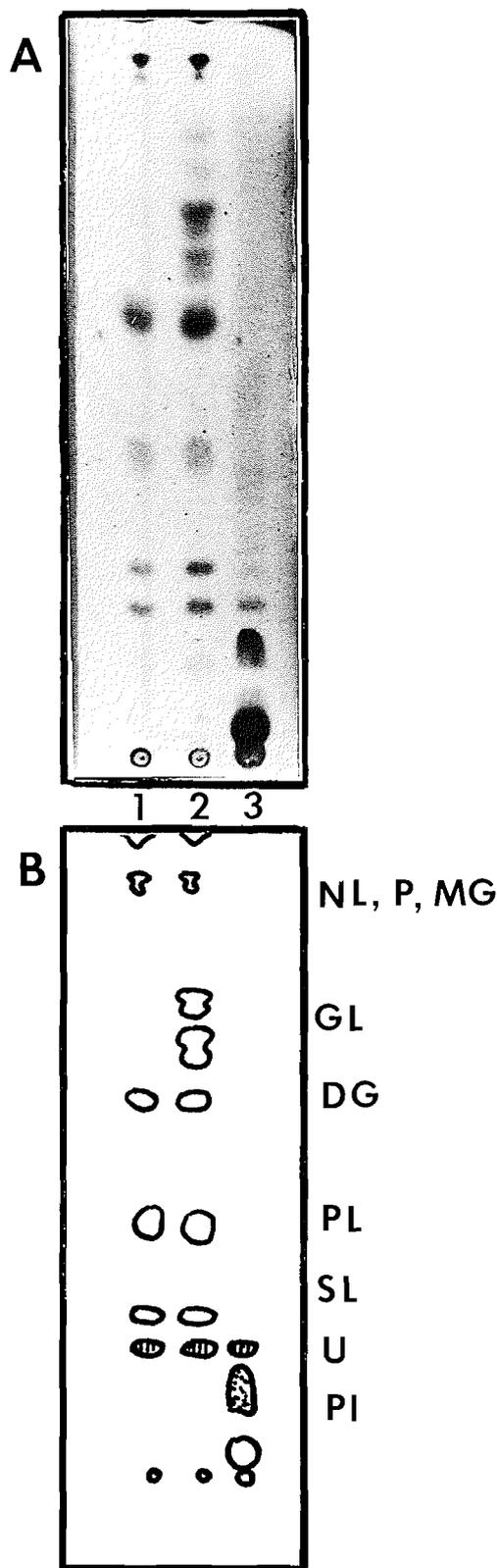
Frankia–Anabaena *comparison*

Comparisons were made between *Anabaena variabilis* cultures with and without heterocysts and *Frankia* strains CpI1 and ArI3 at different morphological stages. The heterocyst-specific glycolipids of *Anabaena* were not seen in any of the *Frankia* extracts, whether they were from sporangium-enriched, filamentous, or vesicle-induced cultures (Fig. 2). An important point to be made is that the fraction of cell mass contributed by vesicles in an induced culture was very small and may have contained vesicle-specific lipids that either were masked by other lipids from the filaments or were not seen at all because they occurred at such low concentrations. A more conclusive answer to the question of vesicle-specific lipids would be obtained by examining the lipids of purified vesicle preparations; to date these are not yet available.

Some *Frankia* extracts did appear to have an unidentified lipid in common with *Anabaena* (Fig. 2). This lipid was previously reported by Nichols and Wood (1968) and travels below the sulfolipid in *Anabaena* and just above PI in the *Frankia* extracts ($R_f = 0.20$) in the solvent system of Nichols and Wood (1968). The unidentified lipid is present in *Anabaena* extracts both with and without heterocysts. Further characterization of this lipid in other solvent systems is in progress.

Sugars

Two sugars, a hexose and a disaccharide, were found in both the chloroform–methanol extracts and the ethanol extracts. The verification of these substances as nonlipid was obtained by two separate methods: (*i*) filtration through Sephadex, using the method of Wells and Dittmer (1963) for the separation of nonlipids from lipid extracts and (*ii*) an acid hydrolysis of the purified compounds which yielded a water-soluble fraction containing a substance with the chromatographic properties of glucose and no lipoidal moiety, confirmed by examination of the petroleum ether fraction (cf. Dittmer and Wells 1969).

All the *Frankia* extracts from all the strains examined contained a disaccharide tentatively identified as trehalose, since it cochromatographed with authentic trehalose in five different paper-chromatography solvent

FIG. 2. TLC of *Frankia* and *Anabaena* total lipids in the solvent system chloroform – methanol – acetic acid – water, 85:15:10:3.7. Visualization: anisaldehyde – sulfuric acid spray. Fig. 2A. TLC plate. Fig. 2B. Tracing of TLC plate. *1*, *Anabaena variabilis* without heterocysts; *2*, *Anabaena variabilis* with heterocysts; *3*, *Frankia* strain ArI3 (filamentous). NL, neutral lipid; P, pigment; MG, monogalactosyl diglyceride; GL, glycolipids; DG, digalactosyl diglyceride; PL, phospholipid; SL, sulfolipid; U, unidentified lipid; PI, phosphatidyl inositol.

systems as well as in the TLC solvent system used (Table 2). When hydrolyzed, this substance yields a hexose with chromatographic properties identical with those of D-glucose. The disaccharide obtained from the extracts used in these tests is nonreducing. No correlation could be found between the occurrence of trehalose in the extracts and the morphological condition of the *Frankia* from which the extracts were made (Fig. 3). All sporangium-enriched, or vesicle-containing, or filamentous cultures appeared to contain similar amounts of the sugar. There was, however, a direct correlation between the occurrence of sugars and growth medium and age. All cultures grown on M6B medium (which includes glucose) contained both the hexose, identified as glucose, and trehalose, except when the culture was over 55–60 days old; the older cultures contained only glucose (Fig. 3). Curiously, extracts made from cultures grown on Qmod medium, which contains glucose, did not yield a glucose spot. An extract made from the lyophilized supernatant of a culture grown on a defined medium contained none of the lipids or sugars found in the other lipid extracts tested.

Discussion

The fact that the phospholipid composition of all the *Frankia* strains we examined (except EUN1f) was consistent is of taxonomic significance. In combination with morphological characteristics and other chemical characteristics such as cell wall type, a description of the genus *Frankia* has begun to emerge. From our observations, the *Frankia* phospholipids were unaffected by changes in medium composition, age of culture, or morphogenesis; this stability makes them more valuable as taxonomic criteria.

The comparison between *Anabaena* and *Frankia* extracts revealed that specific glycolipids like those of heterocysts were not found in *Frankia* filaments or sporangia. Further, no differences were observed in the lipid content of vesicle-induced versus filamentous *Frankia* cultures. This result may be because there were very few vesicles and they did not contribute enough lipid for visualization using TLC or because the purported lipids that make up the specialized vesicle envelope are the same as those found in other structures. Examination of purified vesicle preparations is necessary before it can be determined that *Frankia* has vesicle-specific lipids and that these lipids are the same or similar to those found in *Anabaena* heterocysts. In any event, *Frankia* may have an unidentified lipid in common with *Anabaena*. The unidentified lipid is not restricted to the heterocysts as was determined by comparison of *Anabaena* extracts with and without heterocysts. Further characterization of this unidentified lipid in *Frankia* is necessary to determine if in fact it is the same as the lipid in *Anabaena*.

The identification of the disaccharide found in *Frankia* extracts rests on several facts. Upon hydrolysis, this disaccharide yields a single hexose with chromatographic properties identical with D-glucose. The unhydrolyzed carbohydrate cochromatographs with authentic trehalose in several paper and thin-layer solvent systems and is nonreducing. Under these conditions other common glucose disaccharides such as cellobiose, maltose, and melibiose can be eliminated.

The presence of trehalose in *Frankia* is interesting from a taxonomic point of view since many of the strains we tested are known to have different whole-cell sugar patterns (Lechevalier *et al.* 1982), yet all contained the disaccharide. Although trehalose has not been reported in *Frankia* to our knowledge, it is a common storage product found in yeast, fungi, some vascular plants, and actinomycetes (Elbein 1967). In mycobacteria, trehalose occurs in two forms, lipid bound as part of the cord factor and free. It has been proposed that the free trehalose pool in this case is not a reserve energy material but instead is used strictly for structural purposes (Elbein and Mitchell 1973). In *Streptomyces hygroscopicus* there is evidence that the utilization of trehalose is implicated in the early events of spore germination and may supply the necessary energy for the initial stages (Hay-Ferguson *et al.* 1973).

Trehalose has been found to play a role in certain symbioses. In mycorrhizal associations, the fungus in some cases uses host-derived glucose to synthesize trehalose for its own purposes (Lewis and Harley 1965). In the reverse situation, orchid seedlings, which are obligatory mycorrhizal symbionts, have been shown to utilize exogenous trehalose supplied by the fungus in early growth (Smith 1966, 1967). In this way, trehalose may play an intermediary role as a carbon source between symbionts. Soybean nodules also contain trehalose (Streeter and Bosler 1976). In soybean the trehalose concentration in the nodule is 210-fold or more higher than in other parts of the plant (Streeter 1980). Its role in the nodule, however, is still not clear.

It is intriguing to speculate what role trehalose plays in *Frankia*, both in pure culture and in the symbiosis. Since trehalose disappears from older cultures grown on M6B medium, one must conclude that the sugar is being metabolized in some way. Blom *et al.* (1980) determined that *Frankia* strain AvcI1 does not utilize glucose as a carbon source and Ensign (personal communication) found that strain EAN1 grows poorly when provided with trehalose as a carbon source.

There appears to be no correlation between the morphological state of the *Frankia* cultures and the presence or absence of trehalose. The sugar is probably not exclusively associated with any one morphological structure. Further examination of pure vesicle, sporangial, and filamentous preparations should be made to
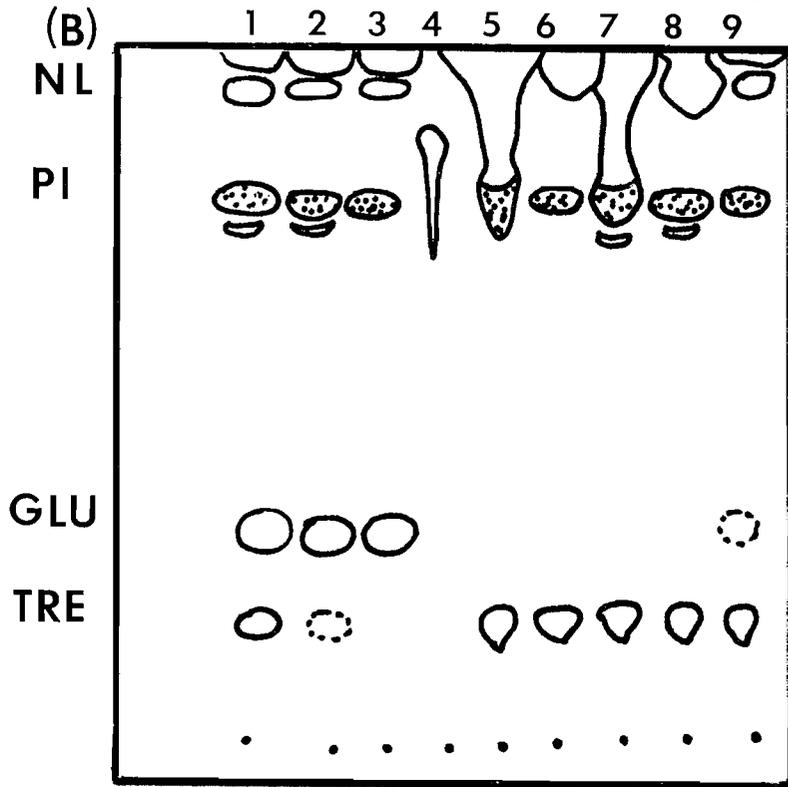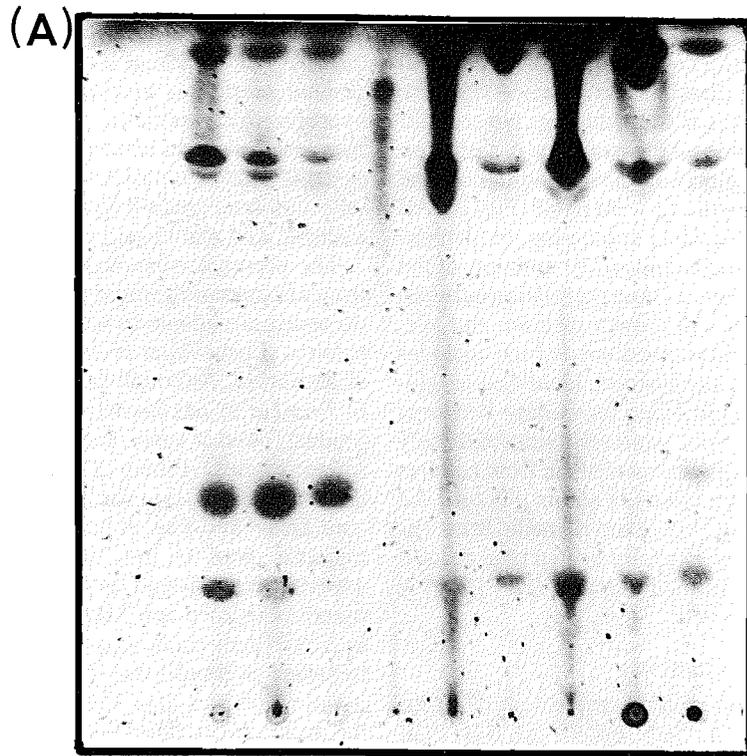
determine whether there are quantitative differences in the amounts of trehalose present in these structures and if in fact trehalose is serving as a localized storage product in *Frankia*.

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FIG. 3. TLC of the total lipids of *Frankia* in the solvent system chloroform–methanol–water, 60:35:5. Visualization: potassium dichromate – sulfuric acid spray. Fig. 3A, TLC plate. Fig. 3B. Tracing of TLC plate. *1*, ArI3, M6B, 15 days old; *2*, ArI3, M6B, 46 days old; *3*, ArI3, M6B, 180 days old; *4*, digalactosyl diglyceride standard; *5*, CpI1, BAP, 14 days old, sporangia; *6*, CpI1, modified BAP, 17 days old, many sporangia; *7*, ArI3, BAP, 14 days old, filamentous only; *8*, ArI3, BP, 10 days old, vesicle induced; *9*, CpI1, M6B, 55 days old, filaments, some sporangia. NL, neutral lipids and DPG; PI, phosphatidyl inositol; GLU, glucose; TRE, trehalose.

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