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In vitro physiological approach to classification of *Frankia* isolates of 'the Alnus group', based on urease, protease and β-glucosidase activities

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Summary Most of the *Frankia* strains isolated from *Alnus* and *Myrica* species are morphologically almost indistinguishable, when grown under standard culture conditions. They form similar vegetative hyphae while sporangia are produced in variable amounts from strain to strain.

Physiological reactions were assessed in order to compare 20 strains isolated from various species of *Alnus* and one species of *Myrica* in Europe and North America. Among invariant negative or positive characteristics, differences in urease, protease and β-glucosidase activities appeared to be of significant value.

Introduction

As numerous *Frankia* strains are now isolated and cultured *in vitro*, methods for classifying and identifying them have to be developed. Morphological criteria, chemotaxonomic and serologic studies, as well as host compatibility traits have been used as classification parameters of these strains. Such techniques allowed *Frankia* isolates to be placed into several large groups[2, 3, 7, 8].

Physiological and biochemical characters have been useful in identifying certain actinomycetes and are included in numerical taxomonic studies[13]. Using such characters, we have therefore attempted to compare 20 strains of *Frankia*, isolated in Europe or North America from *Alnus* and *Myrica* host plants. However, standard methods have to be modified to give reliable and repeatable results when applied to a slow growing actinomycete like *Frankia*, and to date, only a few tests were found useful to differentiate strains belonging to the same chemotype and serotype.

This study compares the urease, protease and β-glucosidase activities of these strains and discusses the validity of these activities as physiological classification parameters.

Material and methods

Test strains

20 strains were studied; 19 were isolated from different species of *Alnus* and 1 from *Myrica pensylvanica* (Table 1). For most of them, strains previously tested for cell chemistry and

serology had shown a whole cell sugar pattern D and belong to serogroup I[1,8]. Two strains at least, one isolated from *Alnus incana* ssp. *rugosa* (AirI2) and the other from *Alnus glutinosa* (AgIIa) differed from the typical *Alnus* isolates.

Cultural conditions

Strains were maintained in static liquid culture on a medium containing (g/l): glucose, 10; casamino acids, 4; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1; trace elements of Q mod medium[6]; and 1 ml/l of the following vitamin stock solution (mg/l): thiamin HCl, 10; nicotinic acid, 50; pyridoxine HCl, 50; pH, 6.8. Tween 80 was added to this basal medium at 0.2% except for two strains (AirI2 and AgIIa), since it had the effect of depressing their growth. Before testing, each strain was grown on one of these two media in a culture tube containing 5 ml of medium, for 5 weeks at 28°C.

Urease test

In 5 week old cultures, growth medium was replaced by 3 ml of a strongly buffered urea broth, where production of ammonia could be detected by pH indicator (Difco B280)[14].

Table 1. Strains used in this study

Strain	Source plant	Source location	WCS[a]	SG[b]	by[c]
Ac21a	*Alnus cordata*	France, Corsica			ULY
Ag10ai	*Alnus glutinosa*	France	D[d]		ULY
Ag10f	*Alnus glutinosa*	France			ULY
Ag10g	*Alnus glutinosa*	France	D		ULY
Ag11a	*Alnus glutinosa*	France	X[e]		ULY
Ag12a	*Alnus glutinosa*	France			ULY
Ag21e	*Alnus glutinosa*	France, Corsica			ULY
Ag24a	*Alnus glutinosa*	France			ULY
Ag24b	*Alnus glutinosa*	France			ULY
Ag24c	*Alnus glutinosa*	France			ULY
Ai15a	*Alnus incana*	France			ULY
AirI1	*Alnus incana* ssp. *rugosa*	U.S.A., VT	D	I[f]	MPL
AirI2	*Alnus incana* ssp. *rugosa*	U.S.A., VT	D	H[g]	MPL
ArI3	*Alnus rubra*	U.S.A., WA	D	I	AB
ArI4	*Alnus rubra*	U.S.A., WA		I	DDB
ArI5	*Alnus rubra*	U.S.A., OR		I	DDB
Av16	*Alnus viridis*	France	D		ULY
AvcI1	*Alnus viridis* ssp. *crispa*	Canada, ONT	D	I	DDB
AcN1[Ag]	*Alnus viridis* ssp. *crispa*	Canada, QUE	D	I	ML
MpI1	*Myrica pensylvanica*	U.S.A., MA	D	I	MPL

[a]Whole cell sugar pattern[9]
[b]Serogroup[3]
[c]Isolated by: AB = Alison Berry, Harvard Forest; DDB = Dwight Baker, Middlebury College; ML = Maurice Lalonde, Laval University; MPL = Mary Lechevalier, Rutgers University; ULY = University of Lyon
[d]Contains glucose
[e]Contains fucose
[f]'Alder' serotype
[g]Heterologous reaction with antisera to CpI1 (Serogroup I) and EuI1 (Serogroup II) antisera.

Colonies were then maintained at 28°C and the time needed for a positive response, indicated by a change in colour from yellow (pH 6.8) to red (pH 8.1 and more alkaline), was recorded daily.

Gelatin hydrolysis test

Proteolytic activity was tested with gelatin as the enzyme substrate. In 5 week old cultures, growth medium was replaced by 0.5 ml of physiological saline and a piece of photographic film (Institut Pasteur 53 861) was added into the culture tube[10]. Colonies were then maintained at 35°C and the time needed for a positive response, indicated by the hydrolysis of the gelatin layer of the film, was recorded daily.

To determine the reproducibility of the results, tests of urease and gelatin hydrolysis were replicated at least twice.

Table 2. β-Glucosidase and urease activities, gelatinolytic activity and sporulation of frankiae isolates

Strain	ESC[a]	URE[b]	GEL[c]	Frequency of sporangia[d]
Ac21a		3	0	0
Ag10ai		3	3	++
Ag10f	+	0	0	0
Ag10g	+	0	0	0
Aglla	+	1	5	0
Ag12a		3	3	++
Ag21e	–	3	0	0
Ag24a		2	4	+++
Ag24b	–	2	4	+++
Ag24c	–	2	4	+++
Ail5a[e]		1[e]	1[e]	+++[e]
Airl1		2	3	++
Airl2	+	0	5	+++
Arl3	–	3	4	+++
Arl4	–	2	2	+
Arl5	–	1	3	++
Av16a		2	4	+++
Avcl1	–	3	2	+
AcN1[Ag]	–	3	4	+++
Mpl1	-	5	1	0

[a, b, c]enzymatic activities were evaluated as follows,

[a]esculine hydrolysis:

+ β-glucosidase positive; – β-glucosidase negative

[b]urease activity:

0 negative reaction within 1 month; 1 positive reaction after more than 12 days; 2 positive reaction between 8 and 12 days; 3 positive reaction between 5 and 8 days; 4 positive reaction between 2 and 5 days; 5 positive reaction within 2 days

[c]gelatinolytic activity:

0 negative reaction within 1 month; 1 positive reaction after more than 10 days; 2 positive reaction between 7 and 10 days; 3 positive reaction between 4 and 7 days; 4 positive reaction between 2 and 4 days; 5 positive reaction within 2 days

[d]the frequency of sporangia was evaluated visually:

0 no sporangia; + a few small sporangia; ++ intermediate frequency; +++ numerous and large-size sporangia

[e]Ail5a became available late in this study and grew poorly on all media tested.

Esculin hydrolysis test

A 5 week old colony was used to inoculate an agar medium supplemented with 0.1% esculin and 0.1% ferric citrate (Institut Pasteur 54 310). The esculin hydrolysis, due to a β-glucosidase activity, gives rise to coumarin which react with ferric citrate to form a brownish black complex.

Results and discussion

Urease activity

A large number of bacteria are capable of urease production. Using a strongly buffered urea broth, it was possible to differentiate strains producing large or small quantities of ammonia by urea hydrolysis and we suggest a graded rate response in their ability to hydrolyze urea under the conditions used (Table 2). Three strains (Ag10f, Ag10g and AirI2) were urease negative; in contrast, MpII exhibited a particularly strong positive reaction.

These reactions were consistent through repeated testing, and the variations never exceeded two days for the strain AcN1^{Ag} tested five times over the year. Nevertheless, a good standardization has been

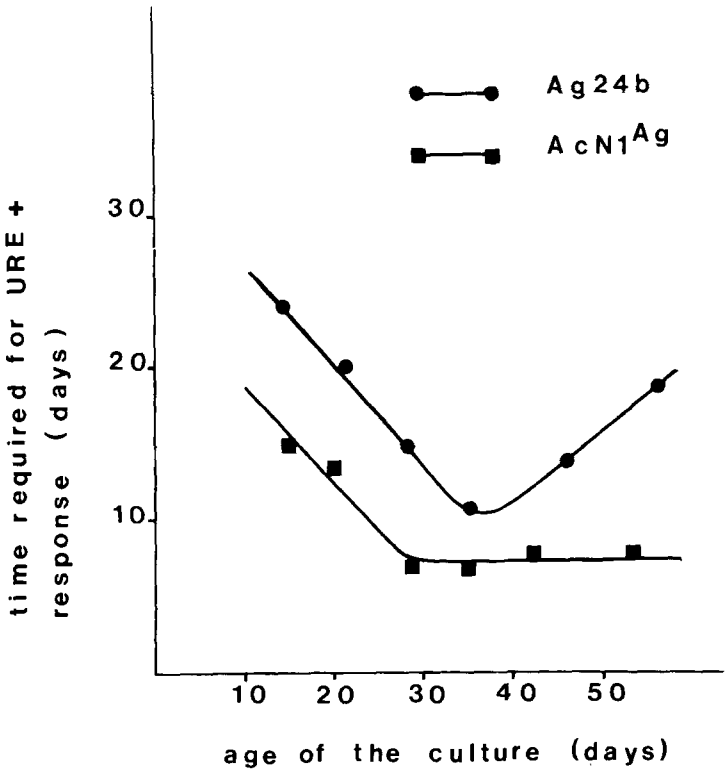

Fig. 1. Influence of the age of the culture on the urease activity of two strains of *Frankia* (Ag24b and AcN1^{Ag}).

found necessary to give reliable results. Not only the cultural conditions, but also the age of the culture may markedly influence the urease production (Fig. 1). In our experiments, the sensitivity of urease activity to the age of the culture depended on the tested strains. After a phase of increasing reaction during the first month following the inoculation, this activity remained constant for $AcNl^{Ag}$, whereas it decreased drastically for Ag24b. Thus, two tests, performed on one resp. two months old cultures, would appear to be of better use to differentiate some strains from each other. Notwithstanding the difficulties in standardization, urease tests have been shown useful in identification of actinomycetes[11, 12].

Proteolytic activity

Gelatin was used as the enzyme substrate, because gelatin hydrolysis tests are very easy to perform, and because gelatin is very susceptible to pronase and is degraded by a lot of bacteria for which proteolytic activity has been revealed[15].

Pronounced differences in the ability of the tested strains to produce a protease capable of digesting gelatin under our conditions were observed (Table 2). AirI2, which has already been shown to produce caseinase[7], presented the strongest activity; in contrast, Ac21a, Ag10f, Ag10g and Ag21e gave negative to very weak results. The hydrolysis of gelatin was positively correlated with presence of numerous sporangia in the colonies, except for the strain Aglla (Table 2). A possible explanation is that extracellular proteases are mostly synthesized during sporogenesis. Similar differences between protease synthesis during growth and sporogenesis have been observed and studied for the genus *Bacillus*[4]. Both the ability of producing sporangia and the protease activity of each strain were not affected by numerous transfers over the year, under the conditions used.

β-Glucosidase activity

Most of the tested strains were not able to hydrolyze esculin and are, therefore, considered as β-glucosidase negative. However, Ag10f, Ag10g, Aglla and AirI2 were β-glucosidase positive (Table 2).

Validity of urease, protease and β-glucosidase activities as physiological classification parameters

On the basis of both chemotaxonomic and serologic results, all of the tested strains, except Aglla and AirI2, were so far indistinguishable. Physiologically, they are strictly microaerophilic, have rather similar carbohydrate and lipid-utilization pattern[7] and they could only be distinguished by nitrate-reductase activity[8]. With standard methods,

urease, gelatin and esculin hydrolysis tests gave reliable and reproducible results when applied to various *Frankia* isolates. These results prove urease, protease and glucosidase activities as useful criterions in the differentiation among strains isolated from *Alnus* or *Myrica* host plants, since there were important variations in urease, protease and β-glucosidase production from one strain to another.

The most significant results of this survey appear to be the very strong urease activity of the strain MpII, and the β-glucosidase activity together with no urease activity of the two strains Ag10f and Ag10g. The strains Aglla and AirI2, which have already been separated from the 'typical *Alnus* isolates', were shown once more physiologically different.

The described tests can be a useful addition to the methods previously used in differentiating frankiae isolates.

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