Grouping of *Frankia* strains on the basis of susceptibility to antibiotics, pigment production and host specificity

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Thirty-nine selected *Frankia* strains belonging to different genomic species were clustered on the basis of their *in vitro* susceptibility to 17 antibiotics, pigment production and ability to nodulate plants of the genus *Alnus* and/or the family Elaeagnaceae, or the family Casuarinaceae. The majority of the strains studied fell into three cluster groups, A, E and C, corresponding to the three host-specificity groups, *Alnus*, *Elaeagnus* and *Casuarina*. Within the groups, eight composite clusters, consisting of at least two strains, and five single-member clusters were recovered at the 0.74 distance level, in good agreement with levels of genetic relatedness between the strains. In addition, five strains were recovered as single-member clusters not in the cluster groups, four of them representing single-member genospecies and one strain not assigned to any known genospecies. The concordance between the phenotypic clusters and the genospecies described previously shows that the grouping may reflect the taxonomic structure of the genus *Frankia*. For some clusters, differentiating phenotypic characters were found which may be useful for species definition.

Keywords: *Frankia*, phenotypic clusters, differentiating characters

INTRODUCTION

Actinomycetes of the genus *Frankia* are nitrogen-fixing root nodule symbionts of diverse non-legume dicotyledonous plants (for a review, see Benson & Silvester, 1993). The first attempt to classify these micro-organisms was undertaken in the absence of pure cultures. In 1970, Becking described 10 species of *Frankia*, based on the source plant and the morphology of endophytes within root nodules. Since the first isolation of a *Frankia* strain in pure culture (Callaham *et al.*, 1978), a large number of strains have been isolated and studied *in vitro* and it has become evident that the previous taxonomic system (Becking, 1970) is not correct. Numerous attempts have been made to divide *Frankia* isolates into groups on the basis of their morphology, physiology, cell chemistry, etc. (for a review, see Lechevalier, 1994) and some potentially useful taxonomic characters have been revealed. However, the relationships of the resulting groups have been difficult to discern because the strains studied

were often few and different from study to study. Host plant responses have been used to group *Frankia* strains into three major host-specificity groups (Normand & Lalonde, 1986; Baker, 1987; Torrey, 1990). The *Alnus* group includes strains able to nodulate *Alnus* (Betulaceae), *Myrica* and *Comptonia* (Myricaceae). The *Elaeagnus* group includes strains infective on *Elaeagnus*, *Hippophaë*, *Shepherdia* (Elaeagnaceae) and *Colletia* (Rhamnaceae). The *Casuarina* group includes strains infective on *Casuarina* and *Allocasuarina* (Casuarinaceae). Lalonde and co-workers proposed a redefinition of two of Becking's species, *Frankia alni* and '*Frankia elaeagni*', to accommodate strains according to their host-specificity group, *Alnus* or *Elaeagnus*, respectively (Lalonde *et al.*, 1988). In addition, *F. alni* has been proposed to be divided into two subspecies, *pommerii* and *vandijkii*, based on the ability of strains to sporulate in root nodules.

Since then, a total of 19 genomic species have been delineated among *Frankia* strains on the basis of their DNA–DNA relatedness (Fernandez *et al.*, 1989; Akimov & Dobritsa, 1992; Lumini *et al.*, 1996), which is currently believed to be the best method to assess

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relationships among strains at the species level (Wayne *et al.*, 1987; Ursing *et al.*, 1995; Vandamme *et al.*, 1996). These include seven genospecies among *Alnus*-compatible strains, 11 genospecies among *Elaeagnaceae*-compatible strains and one genospecies among *Casuarinaceae*-compatible strains. These studies have shown that host specificity is indeed a strong criterion which allows division of *Frankia* strains into large groups; however, these are of supraspecific, rather than specific, rank. Thus, additional species of *Frankia* need to be described. The genospecies delineated have not been described as nomenclatural species, since phenotypic characters have not been available for their differentiation, as proposed by Wayne *et al.* (1987).

A search for differentiating taxonomic characters is exceptionally laborious and time-consuming in the case of frankiae. *Frankia* strains are morphologically similar and cannot be differentiated by this criterion. Physiological characters are more diverse. However, they have been studied on a limited basis and only in a few strains. *Frankia* strains grow very slowly, with generation times up to several days (Akkermans *et al.*, 1992) and are routinely cultivated in liquid media, with only single strains being able to grow on the agar surface. Thus, a very high level of sterility in experiments is required, all the more so since homogenization of mycelium and high inoculum density are needed to obtain subcultures (Baker, 1990). The latter circumstance, moreover, does not practically allow a reliable visual estimation of growth. As a consequence, the ability to grow on various C and N sources, vitamin requirements, effects of growth inhibitors, pH, temperature, water potential, etc., have been typically determined on the basis of biomass increase. This has been estimated, more or less accurately, by measuring dry weight, total organic carbon, total protein, total ATP, optical density of sonicated cultures and packed cell volume, etc. (e.g. Blom *et al.*, 1980; Burggraaf & Shipton, 1982; Shipton & Burggraaf, 1982a, b; Vogel & Dawson, 1986; Dawson & Gibson, 1987; Faure-Raynaud *et al.*, 1990). Altogether, these difficulties preclude extensive physiological searches for differentiating characters, as well as numerical taxonomic analysis of phenotypic traits which has been proved to be a useful tool for delineating bacterial taxospecies. Therefore, computer groupings of *Frankia* strains have only been performed on the basis of chemotaxonomic characters, namely, whole-cell sugar composition (St-Laurent *et al.*, 1987) and fatty acid composition (Simon *et al.*, 1989; Mirza *et al.*, 1991), as well as of isoenzyme patterns (Gardes *et al.*, 1987). However, these studies have included only a few, if any, strains studied by DNA-DNA hybridization and this considerably diminishes the taxonomic value of the results obtained. The above-mentioned problems and others have even culminated in a point of view that the progress in classification of difficult-to-grow *Frankia* spp. is only possible through molecular, not phenotypic, studies (Lechevalier, 1994).

We studied 39 *Frankia* strains with the aim of searching

for easily determined phenotypic characters of possible taxonomic value. Thirty-four strains represented 16 of the 19 genospecies described so far (Fernandez *et al.*, 1989; Akimov & Dobritsa, 1992; Lumini *et al.*, 1996). Most of the other strains (4 of 5) have also been studied by DNA-DNA hybridization (Fernandez *et al.*, 1989) but have not been assigned to genospecies due to incomplete data. The strains were tested for their *in vitro* susceptibility to 18 antibiotics and for pigment production, and these data, combined with previously reported data on host specificity, were used for computer grouping of the strains. This approach resulted in phenotypic clusters corroborating the genospecies delineated within the genus *Frankia* and in revealing differentiating characters for some clusters.

METHODS

Strains. The *Frankia* strains used and their sources are listed in Table 1.

Growth conditions. The strains were routinely grown in submerged liquid cultures at 28 °C for 3 weeks without agitation. The growth media numbered in Table 1 are as follows: 1, 0.5 × QMod/Tween (Blom *et al.*, 1980); 2, P + N (Hafeez *et al.*, 1984) without biotin; 3, FTW (Simonet *et al.*, 1985); 4, QMod without Tween 80; 5, BAP (Murry *et al.*, 1984); 6, OS-1 (Dobritsa & Stupar, 1989); 7, FTW without Tween 80. Pigment production by all strains was observed in P + N medium, as well as in the medium in which the strain grew best (Table 1), if different from P + N. Pigments were categorized into one of four colour groups (Table 2).

Antibiotic susceptibility tests. For antibiotic susceptibility tests, inocula were prepared from 15 ml cultures maintained in the medium in which they grew best. They were concentrated by centrifugation (3000 g, 10 min), washed twice with distilled water, resuspended in 5 ml P + N medium and inoculated into 200 ml P + N medium in 500 ml flasks. After 3 weeks incubation, the mycelium (about 0.1–0.15 ml cell packed volume ml⁻¹) was homogenized in the culture medium with a teflon-pestle glass homogenizer and mixed with an equal volume of P + N medium containing 1.5% (w/v) Bacto-agar (Difco) which had been melted and maintained at 45 °C. The cell suspension containing 0.75% agar was used to overlay P + N plates containing 1.5% Bacto-agar (3 ml per 90 mm plate or 4.5 ml per 110 mm plate). After the top layer had solidified, antibiotic disks (Difco) were placed aseptically onto the agar in the centre (one disk per plate). The dishes were sealed with Parafilm and incubated at 28 °C. After 14 d incubation, growth of strains was compared with that on control plates without antibiotics and inhibition zone diameters around the disks were measured. Disks containing ampicillin (10 µg per disk), bacitracin (10 U), carbenicillin (100 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (2 µg), nalidixic acid (30 µg), neomycin (30 µg), novobiocin (30 µg), penicillin G (10 U), polymyxin B (300 U), rifampicin (5 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg) were used.

Coding of data. There were 21 rows (characters) and 39 columns (strains) in the final data matrix. Seventeen characters were derived from the antibiotic susceptibility data (excluding the invariable character of susceptibility to nalidixic acid). The mean inhibition zone diameter (mm) for

Table 1. *Frankia* strains used and their sources

Strain	Original host	Geographic origin	Reference	Growth medium*
<i>Alnus</i> host-compatibility group				
Cp11	<i>Comptonia peregrina</i>	Massachusetts, USA	Callaham <i>et al.</i> (1978)	1
Ar13	<i>Alnus rubra</i>	Oregon, USA	Berry & Torrey (1979)	1
Avc11	<i>Alnus viridis</i> subsp. <i>crispa</i>	Ontario, Canada	Baker <i>et al.</i> (1979)	1
Avc11.R1	<i>Alnus glutinosa</i>	The Netherlands	Akimov & Dobritsa (1992)	1
Ar14	<i>Alnus rubra</i>	Washington, USA	Baker <i>et al.</i> (1981)	1
Air11	<i>Alnus incana</i> subsp. <i>rugosa</i>	Vermont, USA	Lechevalier <i>et al.</i> (1983)	1
An2.1	<i>Alnus nitida</i>	Swat, Pakistan	Hafeez <i>et al.</i> (1984)	2
An2.24	<i>Alnus nitida</i>	Swat, Pakistan	Hafeez <i>et al.</i> (1984)	2
Ai1c	<i>Alnus incana</i>	Hämeenlinna, Finland	Weber <i>et al.</i> (1988)	3
Ai15a	<i>Alnus incana</i>	Hämeenlinna, Finland	Weber <i>et al.</i> (1988)	3
Ag9b	<i>Alnus glutinosa</i>	Hämeenlinna, Finland	Weber <i>et al.</i> (1988)	3
Ai6b	<i>Alnus incana</i>	Helsinki, Finland	Weber <i>et al.</i> (1988)	3
Ag15	<i>Alnus glutinosa</i>	Oostvoorne, The Netherlands	Hahn <i>et al.</i> (1989)	2
ARgP5 ^{AG}	<i>Alnus rugosa</i>	Québec, Canada	Normand & Lalonde (1982)	2
Mg60 ₂ ^{AG}	<i>Alnus glutinosa</i>	Landes, France	Fernandez <i>et al.</i> (1989)	3
Air12	<i>Alnus incana</i> subsp. <i>rugosa</i>	Vermont, USA	Lechevalier <i>et al.</i> (1983)	4
<i>Elaeagnus</i> host-compatibility group†				
E1	<i>Elaeagnus</i> hybrid	Camaldoli, Italy	Bosco <i>et al.</i> (1992)	5
E3	<i>Elaeagnus</i> hybrid	Camaldoli, Italy	Bosco <i>et al.</i> (1992)	5
Ean1157	<i>Elaeagnus</i> hybrid	Camaldoli, Italy	Margheri <i>et al.</i> (1985)	5
Cc1.17	<i>Colletia cruciata</i>	The Netherlands	Hafeez <i>et al.</i> (1984)	2
S14	<i>Shepherdia argentea</i>	Samara, Russia	Dobritsa & Stupar (1989)	6
S15	<i>Shepherdia argentea</i>	Samara, Russia	Dobritsa & Stupar (1989)	6
S13	<i>Shepherdia argentea</i>	Samara, Russia	Dobritsa & Stupar (1989)	6
Hr5-o	<i>Hippophaë rhamnoides</i>	Pushchino, Russia	Dobritsa <i>et al.</i> (1990)	2
ORS 060501	<i>Colletia spinosissima</i>	Rio Tercero, Argentina	Gauthier <i>et al.</i> (1984)	5
EAN1 _{pec}	<i>Elaeagnus angustifolia</i>	Ohio, USA	Lalonde <i>et al.</i> (1981)	7
GFN14	<i>Hippophaë rhamnoides</i>	China	Fernandez <i>et al.</i> (1989)	5
K1510	<i>Elaeagnus griffithii</i>	Yunnan, China	Du & Baker (1992)	5
D11	<i>Casuarina equisetifolia</i>	Dakar, Senegal	Gauthier <i>et al.</i> (1981)	5
Ea1 ₁₂	<i>Elaeagnus angustifolia</i>	Ecully, France	Fernandez <i>et al.</i> (1989)	7
Ea111	<i>Elaeagnus argentea</i>	Pushchino, Russia	Sharaya <i>et al.</i> (1985)	6
D-Ea111‡	<i>Elaeagnus argentea</i>	Pushchino, Russia	S. V. Dobritsa, unpublished data	2
D-Ea110‡	<i>Elaeagnus argentea</i>	Pushchino, Russia	S. V. Dobritsa, unpublished data	2
Ea50 ₁	<i>Elaeagnus angustifolia</i>	Mions, France	Fernandez <i>et al.</i> (1989)	7
HRN18a	<i>Hippophaë rhamnoides</i>	Alps, France	Moiroud & Faure-Raynaud (1983)	7
Eu11b	<i>Elaeagnus umbellata</i>	Massachusetts, USA	Baker <i>et al.</i> (1979)	1
<i>Casuarina</i> host-compatibility group				
CeD	<i>Casuarina equisetifolia</i>	Dakar, Senegal	Diem & Dommergues (1983)	5
K114	<i>Casuarina equisetifolia</i>	Yunnan, China	Shi <i>et al.</i> (1991)	5
All1	<i>Allocasuarina lehmaniana</i>	Florida, USA	Zhang & Torrey (1985)	5

* Defined in Methods.

† Includes strains infective on both *Elaeagnaceae* and *Alnus*.

‡ Spontaneous, better-growing derivatives of strains Ea111 and Ea110, respectively.

a given antibiotic tested against a certain strain was determined from several (at least two) independent tests, rounded off to the nearest mm and the resulting value was used as a quantitative character for the data matrix. When no inhibition zones were observed in all parallel tests and, in fact, mycelial growth could be observed beneath a disk, '0' was used for coding the mean zone diameter. If small inhibition zones were observed in some and not in other

parallel tests for a given antibiotic/strain combination, zone diameters in those tests that showed no inhibition zones were assumed to be equal to 6 mm (diameter of an antibiotic disk). Pigment production was coded as binary data: 1 for + and ± and 0 for – and ∓ (see Table 2). The qualitative multistate characters of host specificity were coded as three independent characters and each was scored + (1) for the character state shown and – (0) for the alternative.

Cluster analysis. Computer analyses of the data were performed on NTSYS-pc software (version 1.70; Applied Biostatistics, 1992). Distances between the strains were calculated on the basis of the standardized data set by using the SIMINT program. Clustering was achieved by using the SAHN program with the unweighted mean linkage (UPGMA) algorithm (Sneath & Sokal, 1973).

RESULTS AND DISCUSSION

A sound classification depends upon reliable characters which separate taxa having a high phenotypic similarity. In an attempt to find criteria to be used in the taxonomy of the genus *Frankia*, we looked for tests which are reproducible, easy to perform and read, routine for bacteriology and least affected by subjective interpretation (Bascomb *et al.*, 1973).

Susceptibility to antibiotics

As the main group of characters, *in vitro* susceptibilities of the *Frankia* strains to 18 antibiotics were studied. Antibiotics acting selectively on certain species have been used for taxonomic purposes in some groups of micro-organisms (Gilardi, 1971; Penner & Preston, 1980; Nikitin *et al.*, 1988; Reva *et al.*, 1995) or for an intraspecific grouping of strains (Scavizzi & Bronner, 1987). Computer programs have been written for identification of micro-organisms, based exclusively on susceptibility to antibiotics (Petralli *et al.*, 1970; Friedman & MacLowry, 1973; Sielaff *et al.*, 1976), since the characters are in good agreement with classical taxonomy. Susceptibility to antibiotics has been shown to be useful for classification and identification of certain actinomycete genera as well (Goodfellow & Orchard, 1974; Gordon & Barnett, 1977; Tsukamura, 1981, 1982, 1988).

Preliminary visual estimation of growth of our cultures in liquid media containing dilutions of antibiotics did not give reliable results (S. V. Dobritsa, unpublished observations). Therefore, subsequent experiments were performed by using a modified agar diffusion method. The *Frankia* strains were grown in a thin layer of semisolid agar on the surface of a solid agar medium which resulted in good growth within an acceptable period of time (2 weeks) and in clear-cut, visually determinable inhibition zones around antibiotic disks. To standardize experimental conditions, the same lot of each antibiotic disk was tested against all strains, since we found that different lots might give different results. All strains were tested on the same medium (P + N agar), even though some grow somewhat better in different media (see Table 1). This simple medium was selected because propionate is the best C source for most *Frankia* strains (Akkermans *et al.*, 1992) and, unlike many other *Frankia* media, it does not contain Tween 80. In the presence of Tween 80, susceptibility of *Mycobacterium* spp. to antibiotics has been shown to become significantly increased (Naik *et al.*, 1989). This is supposed to result from increased cell wall permeability and, hence, diffusion of an antibiotic into

cells. In some cases, increased susceptibility of the *Frankia* strains to antibiotics on media containing Tween 80 has also been observed (S. V. Dobritsa, unpublished data). The susceptibility of each *Frankia* strain to each antibiotic was tested at least twice. In case of ambiguous or clearly unexpected results, additional tests were performed. In Table 2, mean inhibition zone diameters obtained are presented (deviations did not usually exceed 10%).

All of the strains examined displayed resistance to nalidixic acid and this invariable character was excluded from computer analyses. The levels of susceptibility to carbenicillin, novobiocin, tetracycline, vancomycin, streptomycin, ampicillin, erythromycin, kanamycin and penicillin G varied widely for different strains (diam. of inhibition zones, 0–94, 89, 85, 79, 75, 72, 72, 71 and 65 mm, respectively). Susceptibility to bacitracin, gentamicin, chloramphenicol, neomycin, clindamycin and rifampicin varied to a lesser extent (41–86 and 0–42, 33, 29, 27 and 27 mm, respectively). Most of the strains studied were resistant to lincomycin and displayed low levels of susceptibility to polymyxin B (0–17 and 8–17 mm, respectively).

The potential value of antibiotic sensitivity tests in the taxonomy of frankiae can be assessed from the data presented. Consistent and marked differences in *in vitro* antimicrobial sensitivity patterns were observed between strains representing different genospecies, whereas strains belonging to the same genospecies showed, on the whole, more similarity in their susceptibility patterns. For all antibiotics, except nalidixic acid, differences between genospecies were greater than those within individual genospecies. The mean within-genospecies levels of variance ranged from 3.7% (for gentamicin) to 22.7% (for lincomycin) of the whole-sample levels of variance. The absolute maximum of variance was observed between the strains belonging to genospecies 1 in their levels of susceptibility to erythromycin, but even this did not exceed 78% of the total variance of the character. Antibiotic sensitivity tests do, therefore, seem to be of value in the subgeneric classification of the genus. In some cases, there is also good correlation between host-specificity group membership and sensitivity to certain antibiotics. Thus, although the sensitivity patterns are generally heterogeneous and often overlapping within and between the *Alnus* and *Elaeagnus* groups, one character, susceptibility to novobiocin, shows a significant discriminating potential at the group level. A larger number of antibiotic susceptibility characters differentiate the homogeneous *Casuarina* group from the other two groups, as discussed below for cluster C1. Susceptibility to some antibiotics may also reflect other forms of variation in frankiae and adaptation to certain environmental conditions. These relationships are important for understanding the place occupied by these species in microbial and plant-microbial associations.

Susceptibility to antibiotics has been studied pre-

Table 2. Susceptibility to antibiotics, pigment production and host specificity of the *Frankia* strains studied

Strain	Geno-species*	Cluster†	Diameter (mm) of inhibition zone with:‡																Pigment production§					Infectivity¶			
			Am	Bc	Cb	Cl	Cm	Em	Gm	Km	Lm	Na	Nb	Nm	Pm	Pn	Rm	Sm	Tc	Vm	Pigment- ation	Colour	Cellular pigment	Soluble pigment	<i>Alnus</i>	<i>Elaeag- naceae</i>	<i>Casuari- naceae</i>
<i>Alnus</i> host-compatibility group																											
Cp11	1	A1	37	47	47	20	0	58	28	37	0	0	0	23	10	25	14	37	14	54	—	—	—	—	+	—	—
Arl3	1	A1	37	45	47	20	0	58	27	37	0	0	0	22	10	33	14	35	14	52	—	—	—	—	+	—	—
Ave11	1	A1	37	47	48	18	0	59	28	23	0	0	0	22	12	32	14	44	14	58	—	—	—	—	+	—	—
Ave11.R1	1	A1	37	47	48	18	0	58	28	23	0	0	0	22	12	33	14	43	14	53	—	—	—	—	+	—	—
Arl4	1	A1	37	54	48	18	19	67	28	37	12	0	0	23	12	25	16	39	14	51	—	—	—	—	+	—	—
Airl1	1	A1	37	54	55	18	19	56	27	37	12	0	0	22	12	30	16	38	18	57	—	—	—	—	+	—	—
An2.1	1	A2	37	54	50	32	0	11	27	34	0	0	0	24	12	32	15	56	19	54	+	R	+	+	+	—	—
An2.24	1	A2	33	54	49	30	0	11	32	33	0	0	13	24	12	32	14	62	19	56	+	R	+	+	+	—	—
Ailc	P2	A3	44	47	35	0	0	46	30	46	0	0	0	25	16	20	0	54	13	67	—	—	—	—	+	—	—
Ail5a	P2	A3	45	47	33	0	0	46	30	46	0	0	0	25	16	20	0	54	12	66	—	—	—	—	+	—	—
Ag9b	P2	A3	29	47	33	0	0	46	30	46	0	0	0	25	17	22	0	54	13	65	—	—	—	—	+	—	—
Ai6b	P3	A4	52	60	36	0	0	53	25	48	0	0	0	34	13	31	20	53	14	52	—	—	—	—	+	—	—
Ag15	P4	1	0	65	0	0	23	58	31	45	11	0	0	20	10	0	13	36	0	48	±	Y	±	—	+	—	—
ARgP5 ^{AG}	3	2	0	48	11	21	12	38	20	18	7	0	0	20	10	0	12	32	12	0	±	Y	±	—	+	—	—
Mg60 ₂ ^{AG}	?	4	70	61	94	21	27	83	34	53	17	0	0	31	16	65	15	60	17	79	—	—	—	—	+	—	—
Airl2	P5	5	0	46	0	22	0	20	25	50	0	0	0	37	16	0	14	75	20	0	+	O/B	+	—	+	—	—
<i>Elaeagnus</i> host-compatibility group																											
E1	10	E1	35	50	35	24	0	34	25	40	0	0	74	23	12	43	13	46	23	45	+	R	+	—	+	+	—
E3	10	E1	35	60	35	24	0	34	27	40	0	0	75	25	13	44	15	46	25	63	+	R	+	—	+	+	—
Ean1157	10	E1	35	50	43	27	0	42	27	40	0	0	79	23	15	45	15	42	27	47	+	R	+	—	+	+	—
Ce1.17	P7	E1	24	48	31	23	0	20	24	43	0	0	89	21	11	19	15	40	26	60	±	Y	±	—	+	+	—
S14	P8	E2	53	46	56	23	15	30	22	42	0	0	12	23	12	35	23	35	85	68	+	P	—	+	—	+	—
S15	P8	E2	52	46	54	21	13	30	22	42	0	0	12	23	12	35	23	35	85	64	+	P	—	+	—	+	—
S13	P8	E2	72	46	60	25	17	40	24	35	0	0	12	23	12	38	23	33	85	66	+	P	—	+	—	+	—
Hr5-o	P8	E2	41	47	45	22	14	43	24	45	6	0	14	22	13	40	20	60	41	59	+	P	+	—	—	+	—
ORS	?	E2	48	45	49	24	16	38	18	38	12	0	10	20	12	36	21	46	55	67	±	Y	±	—	—	+	—
<i>Casuarina</i> host-compatibility group																											
060501																											
EAN1 _{per}	5	E3	43	55	35	33	14	32	28	57	0	0	11	25	10	25	20	51	55	50	+	R	+	+	—	+	—
GFN14	?	E4	36	61	48	20	13	34	18	38	9	0	22	18	10	23	15	33	27	50	+	R	+	±	—	+	—
K1510	ND	E4	33	58	33	25	15	34	15	37	7	0	9	17	10	33	23	29	42	45	+	R	+	+	—	+	—
D11	?	E4	28	52	42	23	0	24	25	31	0	0	19	18	12	27	27	23	46	42	+	R	+	—	—	+	—
Ea1 ₁₂	4	E5	28	41	45	32	0	65	23	35	0	0	14	18	11	26	20	47	22	58	+	Y	+	—	—	+	—
Ea111	P6	E6	63	82	63	18	12	30	25	24	6	0	31	20	14	57	18	38	17	55	±	Y	±	±	+	+	—
D-Ea111	P6	E6	50	82	63	18	12	30	25	23	7	0	27	20	14	50	20	38	17	55	±	Y	±	—	+	+	—
D-Ea110	P6	E6	57	82	61	16	12	30	25	23	6	0	30	20	14	57	18	38	17	65	+	Y	+	+	—	+	—
Ea50 ₁	8	E7	34	65	56	16	12	42	20	37	11	0	14	19	15	33	9	0	22	50	+	R	+	+	—	+	—
HRN18a	7	E8	50	70	36	21	19	24	42	77	0	0	40	19	12	50	17	35	27	74	+	Y	+	—	+	+	—
Eul1b	P9	3	40	86	35	29	17	60	39	49	9	0	13	43	14	32	15	53	76	53	+	Y	+	+	—	+	—
<i>Casuarina</i> host-compatibility group																											
CeD	9	C1	0	67	0	27	17	60	0	8	15	0	13	18	9	0	0	30	32	62	±	Y	±	—	—	—	+
K114	9*	C1	0	67	0	20	17	60	0	6	15	0	13	14	9	0	0	30	32	58	±	Y	±	—	—	—	+
Al111	9	C1	0	67	0	20	21	60	0	16	11	0	13	15	8	0	0	30	50	62	±	Y	±	—	—	—	+

* Data from Fernandez *et al.* (1989), Akimov & Dobritsa (1992), Bosco *et al.* (1992), Lumini *et al.* (1996) and S. V. Dobritsa (unpublished data). ?, Available DNA relatedness data do not allow assignment to known or novel genospecies; ND, not determined.

† See Fig. 1.

‡ Mean values from 2–5 independent tests. Am, Ampicillin; Bc, bacitracin; Cb, carbenicillin; Cl, chloramphenicol; Cm, clindamycin; Em, erythromycin; Gm, gentamicin; Km, kanamycin; Lm, lincomycin; Na, nalidixic acid; Nb, novobiocin; Nm, neomycin; Pm, polymyxin B; Pn, penicillin G; Rm, rifampicin; Sm, streptomycin; Tc, tetracycline; Vm, vancomycin.

§ +, Strong pigmentation; ±, weak reliable pigmentation; ±, weak doubtful pigmentation (solely in old cultures); —, no pigmentation. For details, see Methods. Colour: R, shades of red, reddish brown and chestnut; Y, shades of yellow, pale-yellow, yellowish brown and greyish yellow; O/B, mycelium orange, spores black; P, pink.

¶ Data from Gauthier *et al.* (1981, 1984), Lechevalier *et al.* (1983), Hafeez *et al.* (1984), Baker (1987), Nazaret *et al.* (1989), Dobritsa *et al.* (1990), Torrey (1990), Bosco *et al.* (1992), Du & Baker (1992), Lumini *et al.* (1996) and S. V. Dobritsa & S. N. Novik (unpublished data).

* Strain K114 has been studied by DNA–DNA hybridization and assigned to a certain genogroup (Shi *et al.*, 1991) but none of its members have previously been shown to belong to genospecies 9. It is assigned to genospecies 9 on the basis of author's unpublished data.

viously in some *Frankia* strains in liquid cultures, regardless of possible taxonomic significance. In particular, two strains have been shown by optical density of sonicated cultures to be resistant to low neomycin concentrations (Faure-Raynaud *et al.*, 1990). Several strains have displayed resistance to high concen-

trations of certain antibiotics (Normand & Lalonde, 1986). However, data from these studies cannot be compared with our results because the strains were grown in rich organic media. In addition, in the latter study growth was estimated visually, which is not reliable in the case of liquid cultures, and a long period

of incubation (60 d) might have resulted in inactivation or decomposition of the antibiotics. Antibiotic susceptibility of 11 *Frankia* isolates from the Rhamnaceae has been determined (Carú, 1993; Carrasco *et al.*, 1995) in liquid BAP medium, which is very similar in its composition to P+N medium used in this study. Growth of cultures in the presence of certain antibiotic concentrations (1, 5 or 10 µg ml⁻¹) was estimated by total soluble protein after 6–12 d incubation. The isolates have been shown to be resistant or moderately resistant to nalidixic acid (0–39% growth inhibition) and susceptible to erythromycin, kanamycin, rifampicin, ampicillin and streptomycin (82–100% inhibition). Susceptibility to lincomycin, chloramphenicol and penicillin G varied, depending upon the strain and antibiotic concentration, in the ranges between 0 and 93, 68 and 100, and 20 and 100% inhibition, respectively. The data from these studies are consistent with our results obtained by the agar diffusion method for a different set of *Frankia* strains. It is interesting that for the control strain, BR, isolated from *Casuarina* sp., 100% growth inhibition has been observed with erythromycin, 81% with penicillin G and 9% with ampicillin, and no growth inhibition has been observed with kanamycin, chloramphenicol, nalidixic acid and rifampicin at 5 µg ml⁻¹. This closely corresponds to the susceptibility patterns obtained in this study for three other Casuarinaceae-specific isolates (Table 2).

Pigment production

The capability of the *Frankia* strains to produce pigments was used as an additional character in this study (Table 2). Some members of the genus *Frankia* are known to produce pigments. There are strains producing red, yellow, orange, pink, brown, greenish and black pigments (Lechevalier *et al.*, 1982). It has been supposed that this variability may be a useful taxonomic character at the species level (Benson & Silvester, 1993) and production of pigments of certain colours has been used as a character, amongst others, for computer identification of bacteria (Bascomb *et al.*, 1973). Both susceptibility to antibiotics and pigment production have been used as characters in an extensive numerical taxonomic study of actinomycetes of the genus *Streptomyces* and related genera (Williams *et al.*, 1983). It has been found, however, that the colour of spores and substrate mycelia, as well as soluble pigment production, are not always taxon-specific. For this reason, as well as because determination of the colour of a pigment is, to a large extent, subjective, we used only the presence or absence of pigment production for the grouping of the *Frankia* strains. Subsequently, differences between strains in the colour of the pigments and in the ability to produce pigments intra- and/or extracellularly, shown in Table 2, may be used as additional differentiating characters.

The presence or absence of pigment production in the strains studied correlated to a significant extent with their groupings on the basis of host specificity. Thus, none of the three Casuarinaceae-specific strains pro-

duced pigments and most (13 of 16) of the *Alnus*-specific strains did not produce pigments. On the contrary, pigment production was characteristic of most (19 of 20) of the Elaeagnaceae-specific strains (Table 2).

Host specificity

As the third group of characters, our own data and literature data on host-specificity ranges of *Frankia* strains were used, as summarized in Table 2. The strains tested belong to the three best-studied host-specificity groups, *Alnus*, *Elaeagnus* and *Casuarina*, with some Elaeagnaceae-specific strains being able to cross-nodulate *Alnus* spp. The host plant of origin and the ability to nodulate within certain host-compatibility groups are known to be, to a great extent, relevant taxonomic characters (for a review, see Benson & Silvester, 1993). Both phylogenetic (Normand *et al.*, 1996) and DNA–DNA hybridization (Fernandez *et al.*, 1989; Akimov & Dobritsa, 1992) data have shown that major groupings of *Frankia* strains at the supraspecific level occur according to their source plant and/or host specificity.

Phenotypic clusters

Fig. 1 shows the results of cluster analysis of the standardized data on antibiotic susceptibility, pigment production and host specificity of the *Frankia* strains studied. Cluster groups were defined at the 1.25 distance level, while clusters were defined at the 0.74 distance level. These levels were selected after examination of the dendrogram, as they were the lowest which gave clear groupings, correlating with the host-specificity ranges (Table 2) and genospecies, respectively. It should be noted, however, that the latter level is to some extent arbitrary due to missing data on genetic relatedness between members of independently delineated genospecies (Fernandez *et al.*, 1989; Akimov & Dobritsa, 1992; Lumini *et al.*, 1996). Additional DNA hybridization tests may result in widening, to some extent, the limit specified and in changing the cluster structure in three cases of strain groupings occurring at levels close to this limit which are discussed below.

The majority of the *Frankia* strains tested (34 strains) were recovered in three cluster groups (A, E and C) which could be roughly equated with the host-specificity groups *Alnus*, *Elaeagnus* and *Casuarina*. The cluster groups were in turn further subdivided into eight composite and five single-member clusters. Five strains fell into five single-member clusters not included in any of the cluster groups.

Twelve strains representing three genospecies, with levels of genetic relatedness between their representatives equal to 28–30% (Akimov & Dobritsa, 1992), fell into cluster group A. The group was subdivided

Fig. 1. UPGMA dendrogram showing the relationships among the 39 *Frankia* strains studied, derived from cluster analysis of the data presented in Table 2.

into one single-member and three composite clusters in good agreement with the membership of the strains in the corresponding genospecies.

Eight strains of genospecies 1 (Fernandez *et al.*, 1989; Akimov & Dobritsa, 1992) were separated into two clusters, A1 and A2. The distance level between these (about 1.0) significantly exceeded the minimal level observed in this study (0.85) at which representatives of different genospecies united. Thus, our data show that strains An2.1 and An2.24 (cluster A2), despite their high genomic relatedness (81 and 85%, respectively; Akimov & Dobritsa, 1992) to strain Cp11 (cluster A1), might deserve classification as a separate species. This does not contradict usual taxonomic practice which, in the case of inconsistency between phenotypic and DNA-DNA hybridization data, gives priority to the former (Ursing *et al.*, 1995; Vandamme *et al.*, 1996). It has been generally agreed that a taxonomic conclusion should be based on at least two simple tests (Bascomb *et al.*, 1973). In this case, clear differences between strains assigned to clusters A1 and A2 were observed in the characters of pigment production and susceptibility to erythromycin, with additional differences displayed in the levels of susceptibility to chloramphenicol and streptomycin.

It is also noteworthy that the strains of cluster A1,

which are not easy to differentiate by other phenotypic methods, displayed heterogeneity in their susceptibility to antibiotics. Even though some differences in inhibition zone diameters between these strains might be due to insufficient standardization of the inocula, enough differences are evident to be objective. Thus, strains Ar14 and Air11 were distinguished from the remaining strains of the cluster by their susceptibility to clindamycin and lincomycin. Also, strain Ar14 was distinguished by a higher susceptibility to erythromycin, while strains Avcl1 and Avcl1.R1 were distinguished by lower susceptibility to kanamycin.

Cluster A3 contained phenotypically similar strains of genospecies P2 (Akimov & Dobritsa, 1992), isolated in Finland (Weber *et al.*, 1988), which were distinguished from those in clusters A1 and A2 by their resistance to chloramphenicol and rifampicin and certain differences in their levels of susceptibility to carbenicillin, erythromycin, kanamycin and vancomycin. Strain Ai6b, the only member of genospecies P3 (Akimov & Dobritsa, 1992), isolated in Finland (Weber *et al.*, 1988), united with this cluster at a significant distance level (0.88). Strain Ai6b was differentiated from the strains in cluster A2 by its susceptibility to rifampicin, higher susceptibility to bacitracin, neomycin and penicillin G and a lower susceptibility to vancomycin.

Cluster group E contained 19 strains separated into four composite and four single-member clusters, correlating to a significant extent with the genospecies described. It is interesting that all so-called flexible *Elaeagnaceae*-isolated strains, which are infective on both the *Elaeagnaceae* and *Alnus* (see Table 2), fell into cluster group E in accordance with their source of isolation rather than host-compatibility range. However, these did not form a homogeneous group and were recovered in three distinct clusters, E1, E6 and E8.

Cluster E1 included three phenotypically similar flexible strains of Italian origin (Margheri *et al.*, 1985; Bosco *et al.*, 1992) infective on both *Elaeagnus* and *Alnus* and representing genospecies 10 (Lumini *et al.*, 1996). Another flexible strain, Cc1.17, infective on both *Elaeagnaceae* and *Myrica* (Baker, 1987) and representing single strain genospecies P7 (Akimov & Dobritsa, 1992), fell into the same cluster at the 0.73 distance level. This distance is close to the threshold level defined for clusters and, thus, DNA hybridization is necessary to elucidate the status of this strain (genospecies). Strains of cluster E1 could be differentiated from all other test strains by extremely high susceptibility to novobiocin and, unlike most of the strains in cluster group E, resistance to clindamycin.

Cluster E2 included four strains of genospecies P8 (Akimov & Dobritsa, 1992) as well as strain ORS 060501, not assigned to any genospecies (Fernandez *et al.*, 1989). It is interesting that all *Frankia* strains (S14, S15 and ORS 060501) in which linear plasmids have been found (Dobritsa, 1990; S. V. Dobritsa, unpublished data) fell into the same cluster, E2. Strain EAN1_{pec}, a representative of genospecies 5 (Fernandez *et al.*, 1989), was grouped to cluster E2. However, the distance level between it and the other strains, 0.76, slightly exceeded the threshold level for clusters, chosen on the basis of DNA relatedness data. Nevertheless, the distance is close enough to the limit and therefore the status of strain EAN1_{pec}, assigned to a separate cluster (E3), must be defined more precisely by DNA hybridization. The clear differences in the levels of susceptibility to bacitracin, chloramphenicol, carbenicillin, kanamycin, penicillin G and vancomycin between strain EAN1_{pec} and strains in cluster E2 may suggest that the status will not change.

Clusters E2 and E3 were grouped to cluster E4. The nucleus of the latter was formed by strains GFN14 and K1510 whose genospecies affiliation and affinities are unknown, but it may be of significance that both have been isolated in China (Fernandez *et al.*, 1989; Du & Baker, 1992). An atypical isolate from *Casuarina* sp., D11, which does not nodulate members of the *Casuarinaceae* but is infective on *Elaeagnaceae* (Gauthier *et al.*, 1981), fell into this cluster. Our data showed that strain D11 was a typical representative of the group *Elaeagnus*, not *Casuarina*, on the basis of the selection of characters. However, the 0.71 distance level between strain D11 and the other two strains of cluster E4 is close to the delimiting level for clusters. Therefore, the

status and affinities of the strain need to be confirmed by DNA hybridization. Clusters E2, E3 and E4 shared their closest similarities with each other at the 0.85 distance level, but strains within clusters E2 and E4 displayed significant heterogeneity in their susceptibility to antibiotics which makes selection of characters clearly differentiating these clusters from the other clusters of cluster group E impossible. A single-member cluster containing strain Ea1₁₂, representing genospecies 4 (Fernandez *et al.*, 1989), fused to these three clusters at the 0.92 distance level. This strain was characterized by the lowest level of susceptibility to bacitracin among all test strains and by the highest level of susceptibility to erythromycin among those in cluster group E.

Three strains of Russian origin, representing genospecies P6 (Akimov & Dobritsa, 1992; S. V. Dobritsa, unpublished data), fell into cluster E6 despite differences between them in their ability to nodulate *Alnus* and in pigment production (Table 2). They were distinguished from the remaining strains of cluster group E by the lowest levels of susceptibility to kanamycin and, in addition, they displayed the highest levels of susceptibility to bacitracin among all test strains, except Eu11b (single-member cluster 3). The latter, however, could be clearly differentiated from the strains in cluster E6 on the basis of its levels of susceptibility to ampicillin, chloramphenicol, carbenicillin, erythromycin, gentamicin, kanamycin, neomycin, novobiocin, penicillin G, streptomycin and tetracycline. Strain Ea50₁, the only member of genospecies 8 (Fernandez *et al.*, 1989), was recovered in single-member cluster E7 which joined to cluster E6 at a significant distance level (1.06). It could be distinguished from the strains of cluster E6 by the colour of the pigment produced and levels of susceptibility to ampicillin, bacitracin, erythromycin, kanamycin, novobiocin, penicillin G and rifampicin. In addition, strain Ea50₁ was the only one among all the strains tested that displayed resistance to streptomycin. Strain HRN18a, representing single-member genospecies 7 (Fernandez *et al.*, 1989), shared little similarity with any strains of cluster group E and was recovered in a separate single-member cluster, E8. It displayed the highest levels of susceptibility to gentamicin and kanamycin among all test strains and the second highest levels of susceptibility to vancomycin and novobiocin.

Cluster group C included only one cluster, C1, containing three phenotypically similar members of genospecies 9 (Fernandez *et al.*, 1989; S. V. Dobritsa, unpublished data) which could be clearly differentiated from the remainder of the strains. In addition to their ability to nodulate the *Casuarinaceae*, strains of this cluster were distinguished from all other test strains by their resistance to gentamicin and resistance or weak susceptibility to kanamycin, and from most strains by their resistance to the β -lactams (ampicillin, carbenicillin and penicillin G) and rifampicin. Due to these unique patterns, cluster group C was well separated

from groups A and E, even though phylogenetic studies (e.g. Normand *et al.*, 1996) have revealed that *Casuarina*-specific strains are more closely related to *Alnus*-specific strains, while these two groups show less relatedness to strains in the *Elaeagnus* host-specificity group.

Five strains were clearly differentiated from the remainder and did not fall into any of the cluster groups. Each of them was characterized by a unique antibiogram. For instance, strain AgI5 was the only one that showed no susceptibility to tetracycline and only two strains, ARgP5^{AG} and AirI2, displayed resistance to vancomycin, with all other test strains being highly susceptible to this antibiotic. All five strains were recovered in single-member clusters (1–5) among which only two strains, AgI5 and ARgP5^{AG} (clusters 1 and 2, respectively), joined at the 1.15 distance level. Strains EuI1b (*Elaeagnus* group), AgI5, ARgP5^{AG} and AirI2 (*Alnus* group) represent single strain genospecies P9, P4, 3 and P5, respectively. Their phenotypic isolation demonstrated in this study correlates with low levels of genetic relatedness (0–14%) to representatives of the other genospecies (Fernandez *et al.*, 1989; Akimov & Dobritsa, 1992). Also, strain ARgP5^{AG}, the most studied of these strains, has been shown by both phylogenetic (e.g. Normand *et al.*, 1996) and phenotypic (for a review, see Lalonde *et al.*, 1988) studies, including isoenzyme pattern analysis (Gardes *et al.*, 1987), to be quite distantly related to members of genospecies 1 in the *Alnus* infectivity group. Mg60₂^{AG}, the only test strain originating from the plant genus *Myrica* (isolated from *Alnus glutinosa* inoculated with crushed nodules of *Myrica gale*), has not been assigned to any genospecies (Fernandez *et al.*, 1989). It was characterized in this study by the highest or closest to the highest levels of susceptibility to the β -lactams (ampicillin, carbenicillin and penicillin G) and aminoglycosides (gentamicin, kanamycin, neomycin and streptomycin), as well as to clindamycin, lincomycin, erythromycin and vancomycin.

Taxonomic implications

Analysis of the data on susceptibility to antibiotics, pigment production and host specificity resulted in a phenotypic grouping of the *Frankia* strains which showed good agreement with DNA hybridization data. Thus, the clusters formed may be considered to be acceptable taxa at the species level. In some cases, the characters studied clearly differentiate certain groups of strains (genospecies) and therefore could be useful for species descriptions, as discussed above for composite clusters A1, A2, A3, E1, E6 and C1. Also, each of the five strains comprising single-member clusters not in the cluster groups, as well as most of the strains comprising single-member clusters within the cluster groups, are characterized by unique combinations of characters. These peculiarities may, in principle, be used for descriptions of corresponding species. However, strains Ai6b, Ea50₁, HRN18a, EuI1b, AgI5, ARgP5^{AG} and AirI2, recovered here in

single-member clusters, all represent single-member genospecies. Description of a species represented by a single strain is considered to be inadequate (Vandamme *et al.*, 1996), since characters of a single isolate do not reflect the phenotypic diversity of an entire species. For the same reason, additional studies are needed in the cases of genospecies 4 and 5 containing six and three strains, respectively (Fernandez *et al.*, 1989). In this study, these were each represented by a single strain (Ea1₁₂ and EAN1_{pec}, respectively) which does not allow estimation of the phenotypic diversity of these two genospecies. Finally, for some clusters in cluster group E, containing highly diverse strains, additional phenotypic (clusters E2 and E4) and/or DNA hybridization (clusters E1 and E4) tests are needed to define corresponding species.

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