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Identification of Dipterocarpol as Isolation Factor for the Induction of Primary Isolation of *Frankia* from Root Nodules of *Alnus glutinosa* (L.) Gaertner

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In different types of actinorhizal root nodules, primary isolation of *Frankia* from the endophytic state within short periods of incubation is only possible after the addition of root-lipid extracts. Because substances like Tween 80 or lecithin, which stimulate growth during subsequent cultivation, cannot substitute for the root lipids for this effect, it was concluded that a more specific isolation factor was involved. An active compound was isolated from root-lipid extracts by thin-layer chromatography and identified as dipterocarpol (hydroxydammarenone II) by mass spectrometry and by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The identification of dipterocarpol as the active isolation factor was confirmed by biological tests with an authentic sample of dipterocarpol. The isomer hydroxydammarenone I was active as well. Because this is the first indication that triterpenes of this type are involved in symbiotic plant-microbe interactions, some possible explanations are discussed. A hypothesis is based on adaptive differences between the cell membranes of *Frankia* under endophytic and free-living conditions.

Additional keywords: endophyte, nuclear magnetic resonance spectra.

Endophytic microorganisms must evolve different types of adaptations to the environment of a living host-plant cell. Such adaptations can make it very difficult, if not impossible, to start growth under free-living conditions in nutrient media. It has been observed that in many cases, once the difficulties for primary isolation had been overcome, further cultivation was possible on nutrient media with a not excessively complicated composition. This indicates that the difficulties during primary isolation were not caused by special nutritive or metabolic demands, but by the problem of deadapting from the endophytic to the free-living way of life. However, we must be very careful in drawing conclusions, because the differences between the cultivated and the endophytic forms of a microsymbiont or a biotrophic parasite may be the result of a selection process during the primary isolation, so that the cultures only represent a minority as compared with the dominant endophytic population. Even in symbioses where isolation of the microsymbionts appears to be rather easy, such as the *Leguminosae-Rhizobium* symbioses, isolations from the root nodules may only concern the still-multiplying bacteria in the recently infected cells of the nodules. Efforts to cultivate the endophytic bacteroids have led to conflicting results (MacDermott *et al.* 1987). The viability of the bacteroids obviously depends on their state of development and the degree of endophytic adaptations in relation to the occasional host-microbe balance.

Actinorhizal root nodules appear to be especially suitable for the study of these types of problems. Cultures of the microsymbiont *Frankia* have been available only since 1978. When we compare the successful and as yet unsuccessful isolation efforts from actinorhizal root nodules of different plants on different habitats, three different types of results have been described: rapid outgrowth of hyphae from nodule fragments or suspensions on nutrient media; no outgrowth of hyphae, with only occasionally some outgrowth of very few hyphae after incubation periods of 2–3 mo, but rapid and abundant outgrowth of hyphae within 2–3 wk on nutrient media to which a root-lipid extract was added; no outgrowth of hyphae under any circumstances (Quispel and Burggraaf 1981; Quispel *et al.* 1983). The second type is especially interesting and was further studied by Burggraaf (1984). During subsequent cultivations the root-lipid extracts may be further stimulating. This effect may be caused by the presence of very suitable C-sources, which then can be replaced by propionic acid, for example (Blom 1981), or by an additional effect for which the root lipids may be replaced by substances like Tween 80, Triton X-100, or lecithin in concentrations of 5 mg/L. These additions have no effect during primary isolation, not even on media with propionic acid as C-source. This leads to the conclusions that for primary isolation from the endophytic state, a more specific "isolation factor" is involved.

In this paper we will describe the isolation of an active isolation factor from the roots of *Alnus glutinosa* (L.) Gaertner and its purification and identification as the triterpene dipterocarpol. This result not only may be important for further isolations of *Frankia* strains that are more representative for the dominant endophytic population, but may offer new possibilities for studies on molecular interactions that are characteristic for the endophytic way of life.

MATERIAL AND METHODS

Plant material. Nodules were collected from a grove of *A. glutinosa* (L.) Gaertner along the small lake (De Diemen)

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east of Amsterdam, with root nodules of the spore-negative type.

Seeds were collected in early December from trees of the same species near Leiden. Roots were excised from plants cultivated in a modified Crone's solution according to van Dijk and Merkus (1976) at 12 wk after germination. During the last 4 wk the nitrates were omitted from the nutrient solution.

Isolation procedure. Tests for primary isolation of *Frankia* from root nodules were performed with the nodule fragment method of Burggraaf (1984). Freshly collected root nodules were rinsed with water, and the younger parts were excised and washed with soap for 5 min and ethanol 70% for 5 min, rinsed several times with water, and disinfected with OsO_4 1.5% for 1–2 min (a modification of the method of Lalonde *et al.* 1981). After repeated washings with sterile water, the nodules were crushed in a sterile glass tube with sterile glass pestles to obtain small nodule fragments. These fragments were suspended several times in sterile water, sedimented, and the supernatants discarded. Small nodule fragments, which according to their light-brown color had escaped the killing effect of the OsO_4, were sampled with small forceps and placed on petri dishes with nutrient agar.

For nutrient agar we used the basal mineral medium of Shipton and Burggraaf (1982) with the addition of vitamins and trace elements according to Baker and Torrey (1979) and 5 g of casaminoacids and 10 g of agar per medium. About 20 ml of this medium was placed in petri dishes of 10-cm diameter and sown with six small nodule fragments.

Root-lipid extracts, fractions from these extracts, and pure lipids were dissolved in alcohol in a test tube. A few drops of water were added, and the alcohol evaporated in a boiling water bath until the lipids were homogeneously suspended in the water. Then 5 ml of basal medium with 0.8% agar was added and, after autoclaving and cooling to 40° C, added as a top layer on the petri dish with the nodule fragments. After 10–12 days of incubation at 20° C, the nodule fragments were examined with a reversed microscope. If there remained any doubt about the identity of the outgrowing hyphae as *Frankia*, another examination after 3 wk revealed the formation of the highly characteristic sporangia.

Extraction and fractionation of root lipids. The plant roots were excised, frozen in liquid nitrogen, and lyophilized for 48 hr, after which they were stored at room temperature. Before use they were powdered in a mill (Culatti N.V., Schiedam, The Netherlands), and 10 g was extracted with a mixture of chloroform-methanol (1:2 v/v) in a Sorvall homogenizer for 2 min. After repeated extractions with 100 ml of this solvent, the extraction was continued with the same solvent to which 25 ml of H_2O per 100 ml had been added. All extracts were filtered through Whatman 1 paper, combined, and extracted with a 1% NaCl solution in a separation funnel. The chloroform layer was evaporated in a rotary-film evaporator and the residue taken up in 100 ml of the chloroform-methanol solvent. This was called the total lipid extract (TL), of which 1 ml was equivalent to 0.1 g dry weight of the extracted roots (Burggraaf 1984).

The extracts were fractionated by thin-layer chromatography (TLC), first on preparative plates (PSC-Fertigplatten Kieselgel 60, Merck Darmstadt, F.R.G.), then on analytical plates (DC Plasticfolien or Alufolien, with Kieselgel 60). Three solvents were used: A, isopropyl ether-acetic acid (96:4 v/v); B, toluene-tetrahydro furane-diethyl ether (50:5:5 v/v); C, petroleum ether (40–60° C bp) -diethyl ether-acetic acid (90:10:1 v/v). All fractions obtained were finally dissolved in chloroform-methanol (1:2 v/v) so that 1 ml was equivalent to 0.25 mg dry weight of the original root powder.

Chemical analysis. Chemical identification was obtained by mass spectrometry (MS) and nuclear magnetic resonance (NMR). The mass spectra were measured with an AEI MS 902 system. The electron impact ionization was performed with 70 eV electrons.

The 300 MHz ^{1}H NMR and 75 MHz ^{13}C NMR spectra were recorded on a Bruker WM 300 NMR apparatus. Deuterochloroform was used as solvent and tetramethyl silane as internal standard.

RESULTS

Isolation of the active fraction. The *Alnus* grove from which we collected our nodule material for this study has been used by the first author since 1947. The first indications that growth of the endophyte from these nodules occurred after the addition of a root-lipid extract were obtained in 1955. Since then, although different methods were used, no growth during primary isolation was observed in short-term experiments unless root lipids had been added. That the endophyte in the nodules from this grove still need root lipids during primary isolation is shown by data from some recent experiments (Table 1). They confirm the specific character of the isolation factor because Tween 80 or

Table 1. Activity of root lipid extracts, Tween 80, and lecithin on the outgrowth of hyphae from root nodule fragments in three experiments

	Number of root nodule fragments[b] in the experiments of								
	3-5-85			3-19-85			5-22-85		
		Fragments with hyphae			Fragments with hyphae			Fragments with hyphae	
Additions per 10 ml of top agar[a]	Total number	Number	Percent	Total number	Number	Percent	Total number	Number	Percent
No additions	19	0	0	2	0	0	11	0	0
TL 0.5 ml	21	9	43	...	...	...	...	...	...
TL 0.1 ml	15	12	80	...	...	...	...	...	...
Pt 1.0 ml	22	17	73	13	11	85	16	10	62.5
Tween 80 0.1 mg	...	...	...	17	0	0	6	0	0
Lecithin 0.1 mg	...	...	...	17	0	0	...	...	...

[a] TL, total root lipid extract. Pt, enriched fraction.
[b] All contaminated root nodule fragments were discarded.

lecithin had no effect. Table 1 also shows that activity is highly concentrated in an enriched fraction, called Pt. This fraction was prepared on preparative TLC plates with solvent A and consisted of the eluate from the zones between the Rf values 0.4 and 0.7. These zones are characterized by the presence of some slightly yellow pigments with an orange to red fluorescence under UV light (366 nm).

For further fractionation and purification, small samples of this enriched fraction Pt were placed on a series of analytical plates that enable a better separation than the preparative plates. By using solvent A, two zones were observed that were considered to be identical to the fractions P2 (yellow with orange fluorescence) and P5 (greyish green with red fluorescence) as described by Burggraaf (1984). When subsequently using solvent B, another zone was separated from zone P5, which was invisible in visual light but showed a clear bluish-green fluorescence in UV light (366 nm). The purified fraction obtained from this zone was called X5. A direct comparison of the purified fractions P2, P5, and X5 on TLC with solvent A gave Rf values of 0.44, 0.57, and 0.58 and with solvent B, Rf values of 0.37, 0.43 and 0.22, respectively. Further purification of these three dominant fractions was obtained by repeated TLC and alternating use of the solvents A and B. To prevent contamination with substances from the Plasticfolies, we used Alufolies for the last purification steps. After the last purification step, the plates were washed by a run with solvent C, which did not affect the Rf values of the fractions on the plates. During the last TLC on Alufolies with solvent A, all three fractions, considered pure on Plasticfolies, split into two closely adjacent zones. In the case of P2 and P5, these zones were apparently identical and tested together. The two zones from fraction X5 showed some differences in fluorescence, and they therefore were tested separately: X5 (bluish-green fluorescence) and X4 (light green fluorescence).

The activity of the fractions obtained from the zones P2, P5, X4, and X5 on the outgrowth of hyphae from nodule fragments is shown in Table 2. The results are clear. After sufficient purification only the fractions X4 and X5 are highly active and thus contain the isolation factor(s). The differences between the activities of X4 and X5 with and without some other factors may not be regarded as significant and certainly do not indicate any synergistic effect. Another experiment (data not shown) did not indicate any synergistic or even additive effect of the addition of Tween 80 to the fraction X5.

Chemical identification. The active fractions were studied by 300 MHz ^{1}H and 75 MHz ^{13}C NMR spectroscopy and electron impact MS. The comparison of the ^{1}H NMR spectra of the two fractions X4 and X5 showed complete identity with the exception of a few extra signals in X4 that indicate impurities. All further work was done with the fraction X5.

The 300 MHz ^{1}H NMR spectrum revealed that this compound is a triterpene. Eight singlets from quarternary methyl groups were observed, two of which at 1.70 and 1.63 ppm, broadened by long-range couplings, were obviously attached to a double bond (Fig. 1).

The 2D-COSY spectrum showed these signals to couple with the one proton signal at 5.13 ppm from a proton attached to the same double bond. This signal is coupled to a two-proton multiplet at 2.06 ppm, which in turn is coupled to a signal at 1.45 ppm. From these facts the presence of a 4.4 dimethyl but-3-enyl group, common in tetracyclic triterpenes, is established. The signals at 2.51 and 2.42 ppm are from the two protons at C_2, adjacent to a 3-keto group.

The ^{1}H noise decoupled 75 MHz ^{13}C NMR spectrum has 30 singlets showing that the compound has 30 different carbon atoms (the ^{13}C NMR data are summarized in Table 3). The signal at 218.2 ppm shows the presence of a carbonyl carbon atom. The two signals at 132.0 and 124.6 ppm show

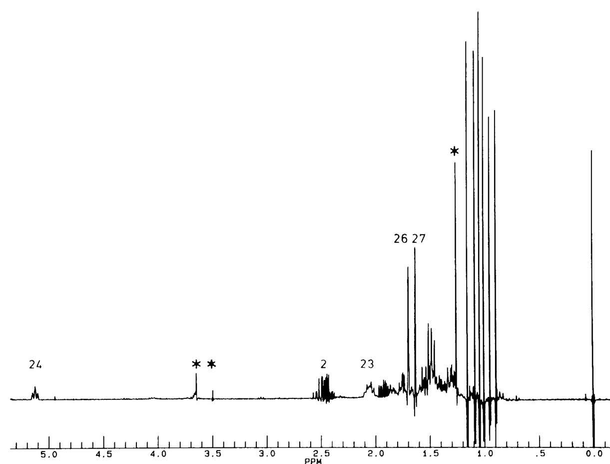

Fig. 1. 300 MHz ^{1}H NMR spectrum of fraction X5. The assignment of some signals is given. The signals indicated with a star are from impurities. The signal at 1.27 ppm is from water.

Table 2. Activity of some purified fractions from root-lipid extracts on the outgrowth of hyphae from root nodule fragments[a]

Additions per 10 ml of top agar	Number of root nodule fragments[b] in a quadruple experiment with four different inoculations								
	A		B		C		D		Mean percent with hyphae
	Total	With hyphae	Total	With hyphae	Total	With hyphae	Total	With hyphae	
No additions	4	0	6	0	6	0	5	0	0
P2 1 ml	5	0	6	0	3	0	4	0	0
P5 1 ml	5	0	6	0	6	0	5	0	0
X4 1 ml	5	5	6	6	6	4	4	4	90
X5 1 ml	2	2	6	3	6	5	6	5	75
X4, X5, each 1 ml	2	1	5	2	3	1	4	4	57
X4, X5, each 2 ml	5	5	6	6	5	4	4	4	95
P2, X4, X5, each 1 ml	5	3	6	2	6	4	3	3	60
P5, X4, X5, each 1 ml	0	0	5	5	5	4	6	6	94

[a] Inoculation date 6-2-86.
[b] All contaminated root nodule fragments were discarded.

that the molecule contains one carbon double bond. The signal at 75.4 ppm shows that the molecule has a carbon linked to an OH group.

From the off resonance proton decoupled spectrum, the presence of seven quarternary carbons is clear (they include the carbonyl carbon, one of the C=C double carbons and the carbon attached to the OH group), five CH groups (one of them is the other double bond carbon), 10 CH_2 groups, and eight CH_3 groups. Based on these facts the structure is $C_{30}H_{50}O_2$.

The EI mass spectrum (AEI MS 902 70 eV) shows the highest mass at m/z 424. This corresponds with $C_{30}H_{48}O$, the mass 18 of H_2O less than $C_{30}H_{50}O_2$. It is known that tertiary alcohols are especially prone to lose the elements of water in the mass spectrometer. The prominent fragment at m/z 127 ($C_8H_{15}O$) shows that the side chain is most likely hydroxylated on C_{21}. The most abundant mass, 109, corresponds to the loss of H_2O from the 127 fragment.

The ^{13}C NMR chemical shift values of X5 were in complete agreement with those of dipterocarpol (hydroxydammarenone II), as published by Asakawa *et al.* (1977). Final proof of the identity of X5 with dipterocarpol (Fig. 2) was attained by comparing with an authentic sample of dipterocarpol supplied by O. Tanaka. He also supplied the 20 epimer of dipterocarpol (hydroxydammarenone I), which showed distinctly different ^1H NMR spectra and the ^{13}C NMR values for C_{21} and C_{22} of 23.5 and 41.8 ppm, respectively, compared with those of dipterocarpol.

Table 3. ^{13}C NMR chemical shift values (δ) of fraction X5

	δ	
1	39.9	t[a]
2	34.1	t
3	218.2	s
4	47.4	s
4α CH_3	26.7	q
4β CH_3	21.0	q
5	55.3	d
6	19.6	t
7	34.5	t
8	40.2	s
8 CH_3	16.0	q
9	50.0	d
10	36.8	s
11	22.0	t
12	25.5	t
13	42.3	d
14	50.2	s
14 CH_3	16.3	q
15	31.1	t
16	27.5	t
17	49.8	d
19	15.2	q
20	75.4	s
21	24.8	q
22	40.4	t
23	22.5	t
24	124.6	d
25	132.0	s
26	25.7	q
27	17.7	q

[a] The s, d, t, and q indicate the multiplicity of the signal in the off-resonance ^1H decoupled spectrum (singlet, doublet, triplet, or quartet, respectively). It indicates that 0, 1, 2, or 3 protons are bound to the carbon.

Biotests with an authentic sample of dipterocarpol. The chemical analyses thus showed that the active fraction X5 contains dipterocarpol. Because there were no indications that other contaminating substances were present in this fraction, it was likely that dipterocarpol is the substance involved in the biological activity as isolation factor during primary isolation from the endophytic state of *Frankia* in this type of nodule material. Proof had to be obtained with authentic samples.

The results of a biotest with the samples of hydroxydammarenone II (dipterocarpol) and its isomer hydroxydammarenone I, obtained from O. Tanaka, are given in Table 4. Both isomers are able to induce growth of hyphae from nodule fragments. Quantitative differences are certainly not significant and do not allow further conclusions. The generally lower percentage of nodules with outgrowing hyphae, as compared with the experiment of Table 2, is merely the result of the nodule test material that

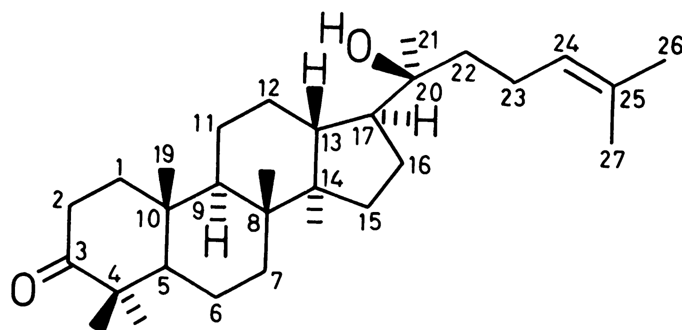

Fig. 2. The structure of dipterocarpol. The numbering is given according to the IUPAC rules for steroids.

Table 4. Effects of hydroxydammarenone I and II (dipterocarpol) on the outgrowth of hyphae from root nodule fragments[a]

	Number of root nodule fragments[b] in a triplicate experiment with three different inoculations						
	A		B		C		
Additions per 10 ml of top agar	**Total**	**With hyphae**	**Total**	**With hyphae**	**Total**	**With hyphae**	**Mean % with hyphae**
No additions	2	0	6	0	6	0	0
Hydroxydammarenone I							
50 μg	10	2	10	2	8	3	25
500 μg	10	5	9	6	10	6	59
Hydroxydammarenone II (dipterocarpol)							
50 μg	11	5	9	3	6	1	43
200 μg	6	6	...	...	...	...	100

[a] Inoculation date 2-16-88.
[b] Contaminated root nodule fragments were discarded.

was collected in winter. We may conclude that dipterocarpol must be regarded to be the active isolation factor in the root-lipid extracts of *A. glutinosa*.

DISCUSSION

In previous experiments it was observed that root-lipid extracts are essential for primary isolation of *Frankia* from certain types of actinorhizal root nodules (Quispel *et al.* 1983). Because this stimulating effect was still observed on nutrient media with propionic acid and could not be replaced by substances like Tween 80 or lecithin, which do stimulate growth during further cultivation (Burggraaf 1984), it was concluded that a more specific isolation factor was required during primary isolation from the endophytic state in this type of nodule material.

After preparative TLC most activity was concentrated in a zone from which the three dominant fractions were isolated and purified by repeated TLC on analytical plates with different solvents. Biological activity was restricted to one of these fractions, which could be identified by MS and ^{1}H NMR and ^{13}C NMR studies as hydroxydammarenone II (dipterocarpol) (Fig. 2). The biological activity of this substance was confirmed with an authentic sample of dipterocarpol. The isomer hydroxydammarenone I, not identified in our root-lipid samples, was active as well.

It remains to be studied whether dipterocarpol or functionally related substances play a role in other samples of actinorhizal root nodules where the isolation of *Frankia* is difficult or only occasionally possible after very long periods of incubation. This is the more important because such isolates then probably are deviating mutants as compared with the dominant endophytic population.

The search for functionally related substances, which may be important in other types of actinorhizal root nodules, is only possible if we have some idea about the function of dipterocarpol in our experiments. This function may be important for our understanding of the molecular plant-endophyte interactions. We therefore want to add some remarks about possible biological functions of dipterocarpol and related substances, which up until now have not been considered as factors in symbiotic plant-microbe interactions.

Dipterocarpol was first isolated from *Dipterocarpus* species (Bandaranayake *et al.* 1975), is a constituent of dammar resins and Ginseng sapogenins (Asakawa *et al.* 1977), and is abundant in galls of *Pistacia terebinthus* (Monaco *et al.* 1973). In roots of *Alnus maximowiczii* a side-chain cyclo derivative was found (Sanada *et al.* 1986), whereas related triterpenes were described in leaves and stems of different *Alnus* species (summarized by Hegnauer 1964). It belongs to the group of ecologically important C-3 oxygenated triterpenoids with a tetracyclic structure, stereochemically different from the structurally related derivatives of tetracyclic phytosterols. The toxic effects of resins, waxes, or saponins with triterpenes such as dipterocarpol (Baas 1985; Kapoor and Chawla 1986; Poehland *et al.* 1987) against mammals, insects, fungi, bacteria, and viruses is of little help in explaining the stimulating effects in our experiments.

Many structurally related steroids are well known for their biological activities, and we must consider the possibility that triterpenes like dipterocarpol show either a similar activity or interfere because of their structural resemblance with such steroids. Steroids are well known for hormonal activities in animals and as plant hormones (Geuns 1982; Mandava 1988). Their effects have been related to the specific regulation of nucleic acid activity. Because the effect of the isolation factor has been observed not only in nodule fragments but in isolated clusters of the endophyte (Burggraaf 1984), an explanation through the interference with plant hormones in the surrounding host-plant cells is unlikely. There are as yet no indications for similar steroid effects on prokaryotes, but such a possibility in the case of *Frankia* certainly cannot be excluded.

Although preliminary experiments did not show any effect of phytosterols like cholesterol, sitosterol, or lanosterol on primary isolation of *Frankia* (data not shown), a possible interference of dipterocarpol with sterols deserves serious consideration. Sterols may have marked, specific effects on growth, sexual and nonsexual reproduction of certain fungi (Nes 1984), and sterol-auxotrophic *Mycoplasmas* (Dahl and Dahl 1983; Kawasaki *et al.* 1985; Rilfors *et al.* 1987). They are ubiquitous as membrane constituents and may affect membrane functions even in low concentrations. In many prokaryotes they are replaced by another type of triterpenoids, the hopanoids (Ourisson *et al.* 1987). Sterols can be directly taken up from the environment, and other triterpenes then can act as substitutes. They then markedly influence membrane fluidity and integrity. Growth responses then reflect the effectiveness of such triterpenes as contributing to membrane function (Nes and Heftmann 1981).

In former experiments it appeared that the stimulation of primary isolation by root-lipid extract was performed as well by extracts of nodule lipids. We thus may conclude that dipterocarpol belongs to the natural environment of the endophyte. Once an endophyte enters the endophytic situation, it is surrounded by substances of the host cells and must rely on these substances for building its cell constituents. The endophytic *Frankia* then must be adapted to the presence of dipterocarpol and may even have incorporated dipterocarpol in the cell membranes. When such endophytic cells are transferred to nutrient media without dipterocarpol, they may lose their integrity unless dipterocarpol is added. Moreover, this hypothesis explains the observation of a gradual adaptation process in which the dipterocarpol during subsequent cultivation may be replaced by less specific membrane-affecting substances like Tween 80 or lecithin until finally, at least on media with propionic acid, no further additions are necessary.

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