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CHELATORS IN CUPRESSACEAE AS IRON TRANSPORT AGENTS FOR *SALMONELLA TYPHIMURIUM*

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Abstract—Strains of *Salmonella typhimurium* which are unable to synthesize their own iron transport agents and require an exogenous chelator were used to examine extracts of the wood of species of Cupressaceae for the presence of iron chelators. Wood from 19 species of five genera were examined and all were found to contain substances that would function as iron transport agents for *S. typhimurium*. The biological activity of most of these species could be explained by the known presence and activity of the thujaplicins. *Juniperus virginiana* and *J. occidentalis* were found to contain a non-tropolone substance that functioned as chelators in *S. typhimurium*. The tropolone nootkatin from *Chamaecyparis nootkatensis* was ineffective as an iron transport agent.

INTRODUCTION

In order to circumvent the limited solubility of iron salts in an aerobic environment microbes have produced a number of ferric-specific chelators called siderophores [1]. The siderophore produced by *Salmonella typhimurium* is the cyclic triester of 2,3-dihydroxybenzoylserine [2]. Mutants of *S. typhimurium* that are unable to produce such compounds will grow well on complex media but for growth to occur on citrate-containing media an exogenous chelator must be supplied [3]. These mutants can be used as a means of detecting chelators in biological extracts and have been used to identify the tropolones, α-, β- and γ-thujaplicin, from the wood of *Thuja plicata* as substances that can participate in the iron transport metabolism of *S. typhimurium* [4]. Other species in the cypress family produce a number of tropolones [5–7] and it is not known if these substances can function as chelators in *S. typhimurium*. Wood from three members of this family were thought to merit special examination for the presence of such compounds: *Juniperus virginiana* and *J. occidentalis* because they do not produce tropolones and *Chamaecyparis nootkatensis* because it contains only the tropolone, nootkatin [5, 6].

RESULTS

Table 1 lists the growth responses of *S. typhimurium* enb-7 to wood samples from various species of cypress. Concentrated hexane extracts, representing as much as 1 kg (fr. wt) of *J. virginiana* wood or 10 g (fr. wt) of *J. occidentalis* wood were tested for tropolones using the iron reagent [8, 9]. No evidence of tropolones was found in either extract (lower limit of detection is about 0.5 mg of tropolone). Extracts of the wood of these species do, however, support the growth of *S. typhimurium* enb-7 (Table 2). When resolved by descending PC the biological activity remained near the origin, with a migration relative to β-thujaplicin (R_β) of 0.0–0.2. There was no evidence (with or without iron reagent) of the characteristic UV fluorescence of tropolones in chromatographs of these extracts [6]. Wood samples from the heart or sapwood of both male and female *J. virginiana* trees tested were found to support the growth of *S. typhimurium* enb-7 (Table 2).

The addition of the aqueous iron reagent [8, 9] to extracts of *Ch. nootkatensis* wood resulted in an intense green color characteristic of a tropolone [8]. The extract was resolved by descending chromatography and examined for biological activity (Table 2).

DISCUSSION

All the wood samples from cypress species that were tested were found to support the growth of *S. typhimurium* enb-7 and TA2700, indicating that the different woods contain iron chelators that will function in this bacteria. The activity in the majority of the wood samples can be explained by the known presence of one or more of the thujaplicins (Table 1). The most interesting situations involve species that do not contain (or contain only one) tropolones.

If tropolones are present in *J. occidentalis* and *J. virginiana* they are present at or below the levels of 0.5 mg/kg and 50 mg/kg of wood, respectively. In *Ch. nootkatensis* extracts, no tropolones other than nootkatin were observed. Large variations in R_β values were observed depending on the chromatogram and solvent preparation methods; however, the relative positions observed for the tropolones were the same as given in the literature [5, 6]. The chromatograms of extracts from *Ch. nootkatensis*, *J. virginiana*, and *J. occidentalis* were examined for biological activity, and activity was found in the regions with R_β 0.0–0.2. Substances with a similar R_β that serve as chelators for *S. typhimurium* have been observed in extracts from *T. plicata* [4]. *S. typhimurium* TA2700, which does not utilize hydroxamic acids [10], grew in the presence of the R_β 0.0–0.2 fraction (from *J. virginiana*, *J. occidentalis* and *T.*

Table 1. Cupressaceae woods as sources of chelators for *S. typhimurium* enb-7*

Species	Source†	Tropolones‡	Growth response§
Chamaecyparis lawsoniana	R, B, Th, Ta	β-Thujaplicin	10–21
Ch. nootkatensis	R, B, Ta, H, Ms, Mh	Nootkatin	3– 9
Ch. thyoides	Z, O	Thujaplicins	5–12
Cupressus arizonica	Z, Th	Several	8–19
C. benthami	Z	Several	10–21
C. glabra	Z	—	5–12
C. lindleyi	Z	Several	2– 7
C. lusitanica	Z	Several	6–10
C. macrocarpa	R	Nootatin	
C. sargentii	Z	Several	6–18
Juniperus deppeana	Z	Several	4– 9
J. monosperma	R	Several	8–15
J. occidentalis	R	None	5–12
J. tlaccida	Z	—	1– 5
J. scopulorum	Z	—	3–10
J. virginiana	R, L, T, O, Th, Ta	None	7–28
Libocedrus decurrens	Z, O, Th	Several	8–19
Thuja occidentalis	R, O, Th	Several	7–14
T. plicata	Ta, C	Several	12–35

*Test substances were applied to the surface of *S. typhimurium* enb-7 lawns as described in the text. Nearly identical results (not shown) were observed with *S. typhimurium* TA2700.

†Sources of wood samples: B = G. M. Barton, Western Forest Products Laboratory, Vancouver, British Columbia; C = commercial lumber; H = A. S. Harris, U.S. Department of Agriculture, Forestry Sciences Laboratory, Juneau, Alaska; L = obtained locally; Mh = P. Moudich, Ketchikan area Tongass National Forest, Ketchikan, Alaska; Ms = E. V. Morris, Pacific Forest Research Centre, Victoria, British Columbia; O = J. R. Olson, Department of Forestry, University of Kentucky, Lexington, Kentucky; R = H. Resch, Department of Forest Products, Oregon State University, Corvallis, Oregon; T = F. W. Taylor, Forest Products Utilization Laboratory, Mississippi State, Mississippi; Ta = M. A. Taras, Department of Forestry, Clemson University, Clemson, South Carolina; Th = R. J. Thomas, Wood Products Laboratory, North Carolina State University, Raleigh, North Carolina; Z = E. Zavarin, Forest Products Laboratory, University of California at Berkeley, Richmond, California.

‡The tropolones reported are from published determinations ([5–8] and references therein). 'Several' means the species contains at least three different tropolones including a thujaplicin. Dash (—) indicates no published tropolone analysis is known.

§The growth is in millimeters from the wood disk. The first figure is the radius of growth inhibition while the second value is the outer limit of growth after 24 hr of incubation at 37°.

plicata). Therefore, if there is a single substance in the R_β 0.0–0.2 fraction with biological activity, it probably is not a hydroxamic acid. When nootkatin was examined for biological activity none was observed.

Several substances are known that function as iron transport agents in one organism but are ineffective in another species; e.g. citrate serves as a siderophore in some strains of *Escherichia coli* [11] but not in *S. typhimurium* [3] and the aspergillic acids support the growth of *Arthrobacter* sp. [12] but not *S. typhimurium* (Table 2).

Normally, there is a low level of bacterial growth throughout the chelator assay plate. Inhibitory substances are evident by a zone of no background growth around the site of sample application. All cedar wood samples tested showed inhibition zones (Table 1) which can be explained by the antibiotic nature of the tropolones [13, 14] or by postulating the presence of toxic substances in the case of *J. vir-*

Table 2. The effect of Cupressaceae wood, extracts, fractionated extracts, and purified substances as iron chelators for *S. typhimurium* enb-7*

Sample/substance	Sample size†	Growth‡
J. virginiana		
Heartwood ♀	0.3	10–17
Heartwood ♂	0.3	9–17
Sapwood ♀	0.2	9–16
Sapwood ♂	0.2	10–19
Extract (complete)	0.8	8–16
R_β 0–0.2	0.8	3–12
J. occidentalis		
Extract (complete)	0.9	8–15
R_β 0–0.2	0.5	3– 9
Ch. nootkatensis		
Extract (complete)	2.0	12–18
R_β 0–0.2	2.0	0– 6
R_β 1.2 (nootkatin)	0.7	None§
Purpurogallin	0.1, 1, or 10 mg	None
Neoaspergillic acid	0.1, 1, or 10 mg	None
Neohydroxyaspergillic acid	0.1, 1, or 10 mg	None
β-Thujaplicin	1 mg	9–19

*Test substances were applied to the surface of *S. typhimurium* enb-7 lawns as described in the text. Nearly identical results (not shown) were observed with *S. typhimurium* TA2700.

†Figures without units are in g of wood or represent an extract or fraction derived from the indicated number of g of wood.

‡The growth is in ml from the wood disk. The first figure is the radius of growth inhibition while the second value is the outer limit of growth after 24 hr of incubation at 37°.

§An inhibition zone of 15 mm (radius) was observed.

giniana and *J. occidentalis*. At low concentrations tropolone and the thujaplicins will function as iron chelators in *S. typhimurium* while at higher concentrations they are inhibitory [4, 13, 14]. Nootkatin was also observed to cause growth inhibition (Table 2).

Only tropolone or tropolones bearing simple alkyl substitutions (the thujaplicins) have been observed to function as iron chelators in *S. typhimurium*. Previously, tropolones (stipitatic acid, stipitatonic acid, and colchiceine) possessing bulky or electron-withdrawing groups were found to be inactive [4]. The tropolones nootkatin and purpurogallin can now be added to this latter list.

Because of the presence of iron transport agents in Cupressaceae, researchers in iron metabolism or microbial virulence should be aware of potential complications [15, 16] because of the frequent use of cedar sawdust as a component of rodent litter.

EXPERIMENTAL

The general chelator assay procedures using *S. typhimurium* enb-7 and TA2700 have been described previously [4]. β-Thujaplicin, aspergillic acids and purpurogallin were obtained from Columbia Organic Co., Dr. J. C. MacDonald, and Aldrich Chemical Co., respectively. Cedar extracts were resolved by descending PC [5, 6]. Chromatograms were developed [4] with *iso*-octane–EtOH (99 : 1). Tropolones were located on chromatograms by UV (254 nm), iron reagent spray [9], microbiological detection [4], and anticipated relative migrations [5, 6].

Wood specimens were obtained from the sources listed in Table 1. Wood disks (6 × 1–2 mm), water or hexane extracts added to 6 mm filter paper disks, or portions of chromatograms were tested for the presence of iron transport agents using lawns of *S. typhimurium* enb-7 or TA2700 on medium E agar [10]. Any microbiological assay that showed visual evidence of contamination was discarded. All operations involving extracts were performed in the dark or in subdued light. All data presented are the averages of at least three separate determinations.

Juniperus virginina wood (14.7 kg of 10–20 cm diameter branches of undetermined sex) obtained locally was cut perpendicular to the grain into 5 mm thick pieces. The sawdust and wood was extracted twice overnight in the dark at 100° with 35 l. H_2O. The H_2O extracts were extracted 4× with *ca* 3 l. portions of hexane. The combined hexane extracts were reduced to *ca* 75 ml of a reddish-brown syrup on a steam cone.

Ch. nootkatensis (*ca* 640 g of 2 × 2 × 40 mm) wood chips were steam-distilled until *ca* 3 l. of distillate was obtained. This distillate was extracted twice with *ca* 400 ml portions of hexane. The hexane fractions were reduced to 3–4 ml of a reddish-purple soln.

J. occidentalis wood chips (95 g of 2 × 2 × 40 mm) were steam distilled until *ca* 1 l. of distillate was collected. After extracting the distillate twice with 200 ml portions of hexane, the hexane fractions were pooled and reduced to *ca* 1.5 ml of an amber soln.

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