

Cutinase Production by *Streptomyces* spp.

Abstract. Forty-five *Streptomyces* strains, including representatives of the plant pathogens *S. acidiscabies*, *S. scabies*, and *S. ipomoea*, were screened for ability to produce enzymes (cutinases) capable of hydrolyzing the insoluble plant biopolyester cutin. Initially, all strains were tested for production of extracellular esterase in liquid shake (250 rpm) cultures at room temperature in defined (glycerol–asparagine) or complex (tryptone–yeast extract with or without addition of mannitol) broth media supplemented with either tomato or apple cutin. Esterase activity was determined by a spectrophotometric assay utilizing the model substrate *p*-nitrophenyl butyrate. Of the five strains exhibiting highest esterase activity, four (*S. acidiscabies* ATCC 49003, *S. “scabies”* ATCC 15485 and IMRU 3018, and *S. badius* ATCC 19888) were confirmed to produce enzymes with cutin-degrading activity (cutinases). Confirmation of extracellular cutinase production was accomplished by use of a new high-performance liquid chromatography method for separation and quantification of released cutin monomers. Monomer identification was confirmed by GC/MS analyses. Cutinase production was induced 2- to 17-fold by inclusion of cutin in the media. To our knowledge this constitutes the first report of cutinase production by *Streptomyces* spp. other than *S. scabies*.

The surfaces of most aerial plant parts including leaves, flowers, and fruits are covered with a protective barrier called the cuticle. The cuticle is made up of two components [11]. The structural component, cutin, is an insoluble biopolymer composed primarily of lipid monomers (C₁₆ and C₁₈ hydroxy and hydroxy, epoxy fatty acids). The cutin is embedded in and covered by a coating of cuticular waxes typically composed of chloroform/methanol-soluble lipids such as hydrocarbons and wax esters. The cuticle is thought to help protect the plant from desiccation as well as microbial and insect attack.

For many years it was uncertain whether plant pathogenic fungi utilized solely physical force to penetrate the cuticle or whether enzymes contributed to penetration. Beginning in the 1970s several plant pathogenic fungi were reported to produce enzymes, cutinases, that hydrolyzed cutin. Several of these fungal cutinases have been purified and characterized. It is now clear that cutinases have an

important role in penetration of intact plant surfaces by certain fungi on the basis of studies with chemical inhibitors, antibodies specific for cutinase, and by use of molecular biology techniques (for a review see [11]). A recent report indicates that fungal cutinase may play a role in determining host tissue specificity [22].

In contrast to the extensive literature on fungal cutinases, very little is known about bacterial cutinases. Prior to our studies, only three bacterial strains had been reported to produce cutinase. Lin and Kolattukudy [14] reported that a strain of *Streptomyces scabies*, the causal agent of potato common scab [10], produced a cutinase when grown in minimal medium with apple cv. Golden Delicious cutin as the sole carbon source. The cutinase was purified and found to be similar to fungal cutinases in molecular weight, amino acid composition, and carbohydrate content. Cutinase production by additional strains of this bacterium and the possible role of cutinase in pathogenicity of this bacterium were not determined. However, the ability of fungal cutinase to degrade the plant biopolymer suberin [5] led to the hypothesis that cutinase production by *S. scabies* may be required to

breach the suberized periderm layer of potato tubers [15]. The other plant pathogen reported to produce cutinase is *Pseudomonas syringae* pv. *tomato*, a leaf pathogen of tomato [1]. This cutinase was not studied in detail, and its role in pathogenicity is not known. In a separate screening study the ability of *P. syringae* pv. *tomato* to produce cutinase could not be confirmed [6]. However, out of the 232 nonfilamentous saprophytic, plant pathogenic, and opportunistic plant and human pathogenic bacteria tested, we detected a cutin-inducible esterase with cutinase activity produced by four strains [6].

The best characterized bacterial cutinase is produced by a plant epiphyte identified as a variant strain of *P. putida* [17, 18]. This cutinase is an "active serine" enzyme, as are the fungal cutinases, but differs from fungal cutinases in amino acid composition and is more stable at high temperatures [17, 18]. The bacterium was isolated from a plant leaf in an apparent mutually beneficial relationship with a nitrogen-fixing *Corynebacterium* sp. It was hypothesized that the *P. putida* strain provided a carbon source for both bacteria by hydrolysis of cutin at the leaf surface, while the other bacterium supplied a source of fixed nitrogen [18].

The goal of the work reported here was to determine whether cutinase production is common among strains of *Streptomyces* capable of causing disease on potato and sweet potato as well as saprophytic *Streptomyces*. The taxonomic classification of *Streptomyces* capable of causing scab diseases of potato has been unsettled, and *S. scabies* was not listed in the approved lists of bacterial names published in 1980 [20]. It is now clear that several genetically distinct types of *Streptomyces* can cause potato scab, and these have been placed into three species [8, 12, 13, 20].

Lambert and Loria [12] recently revised the taxonomic classification of *S. scabies* with a description consistent with the original. Lambert and Loria [13] also recently described a new acid-tolerant *Streptomyces* species (designated *S. acidiscabies*) that attacks potato. The third group of pathogenic strains are highly diverse and have tentatively been designated as *S. albidoflavus* [8]. Because of this confusion, most strains that were received by us under the taxonomic classification *S. scabies* are referred to as *S. "scabies"* until they are reexamined in accordance with the revised taxonomic description.

Materials and Methods

Bacterial strains. The following strains were obtained from the American Type Culture Collection, (Rockville, Maryland): *Streptomyces acidiscabies* ATCC 49003 type strain; *S. badius* strains ATCC 19888 (type strain) and ATCC 39117; *S. scabies* ATCC 49173 (type strain); *S. "scabies"* strains ATCC 3352, ATCC 10246, ATCC 15485, and ATCC 33282; and *S. viridisporus* strains ATCC 27479 (type strain) and ATCC 39115. *Streptomyces ipomoea* strains ISP 5383, Clark Standard, Clark 78-49, Clark 78-51, Clark 78-52, Clark 78-60, Clark 78-61, and Clark 78-62 and *S. "scabies"* strains IMRU 3018 (= ISP 5078), IMRU 3029, IMRU 3647, IMRU 3648, IMRU 3649 were obtained from Dr. Mary Lechavalier, Rutgers University (New Brunswick, New Jersey). *S. "scabies"* NRRL strains B391, B1231, B1514, B1515, B1715, B2187, B2794, B2795, B2796, B2797, B2798, B2799, B2800, B2801, B2802, B3166, B3262, B3637, B12085, B16515, B16516, and B16517 were obtained from Dr. D. Labeda (USDA National Center for Agricultural Commodity Utilization Research, Peoria, Illinois).

Bacterial strains were routinely cultured for short-term maintenance on Difco ISP Medium 4 agar (*S. ipomoea*) or glycerol-asparagine (GA) agar [19] (all others) and stored at 4°C. For long-term storage, filaments and spores were scraped from the surface of cultures grown on solid media, suspended in 50 mM MOPS buffer, pH 6.8, containing 20% (vol/vol) glycerol, and stored at -80°C. Alternatively, the spores and filaments were used to inoculate tryptone-yeast extract (TYE) broth [19] supplemented (*S. ipomoea*) or not supplemented (all others) with mannitol (2%, wt/vol), and the resulting growth was used for long-term storage as described above.

Preparation of cutin. Cutin was prepared from mature fruits of apple cv. Golden Delicious and tomato according to published procedures [23] with slight modification [7]. Pectinase and cellulase were obtained from Sigma Chemical Co. (St. Louis, Missouri).

Screening for esterase and cutinase activity. Sterile culture tubes (20 × 150 mm) containing 5 ml of broth (either GA or TYE with or without mannitol) media supplemented with 0.4% (wt/vol) of cutin were inoculated with 0.1 ml of bacterial stock culture. Cultures were shaken at room temperature at 250 rpm on a rotary shaker. Samples of culture fluid (100 µl) for esterase determinations were typically removed at 3, 8, and 14 days. For cutinase assays, 1 ml of culture fluid was removed at day 8. Culture fluids were clarified by centrifugation in a microfuge at 4°C and either immediately used for assays or stored at -20°C until used.

General esterase activity was determined by a spectrophotometric assay with *p*-nitrophenyl butyrate (PNB) (Sigma) as substrate [18]. The assay mixture (1 ml total volume) contained 2.3 mM PNB and 0.2% (vol/vol) Triton X-100 (Sigma) in 50 mM potassium phosphate buffer, pH 8.0. From 1 to 100 µl of culture fluid was added, and the change in absorbance at 405 nm was monitored for 1 min during which active preparations showed a linear increase in $A_{405\text{ nm}}$. The rate of reaction was determined by reference to a standard curve prepared by adding various amounts of *p*-nitrophenol (the colored reaction product) to the assay mixture. Standard curves were prepared daily.

For cutinase assays, 0.5 ml of culture fluid was added to 30 mg of apple cv. Golden Delicious cutin and 0.5 ml of 0.1 M potassium phosphate buffer, pH 8.0, contained in a screw capped glass test tube with a glass bead (3 mm) to facilitate mixing. The nonionic detergent *n*-octylglucoside (0.1 ml) was added to some assay mixtures to a final concentration of 35 mM. Tubes were shaken (125 oscillations/min) for 18 h in a water bath held at 27°C. After incubation, the resulting solution was acidified with acetic

acid, and released cutin monomers were extracted with chloroform-methanol according to the method of Bligh and Dyer [2]. The organic soluble material was dried under a stream of nitrogen and taken up in 1 ml of chloroform : methanol (85 : 15, vol/vol). The samples were filtered through glass wool, dried under a stream of nitrogen, and taken up in 1 ml chloroform : methanol as above. Controls consisted of broth media alone, after removal of cutin by centrifugation, and buffer alone.

The released cutin monomers were separated and quantified by high-performance liquid chromatography (HPLC). The methodology used was a modification of an HPLC technique developed in this laboratory to separate all plant lipid classes [16] and is the subject of a separate report [7]. Quantification of the cutin monomers was based on a standard curve of area units versus weight prepared for a sample of 9,10,18-trihydroxyoctadecanoic acid, which comprised about 15% of our apple cutin preparations [7].

The chemical structures of the released cutin monomers were determined by gas-liquid chromatography/mass spectrometry (GC/MS). The extracted cutin monomers were converted to the methyl esters by reaction with boron trifluoride in methanol (Pierce) according to the manufacturer's instructions. Hydroxyl groups were silylated with N,O-bis(trimethylsilyl)acetamide for 30 min at room temperature. The silylated methyl esters were examined by GC/MS on a Hewlett-Packard model 5990B instrument fitted with an Ultra 1 (methyl silicone, Hewlett-Packard) 12 M capillary column using temperature programming from 125–250°C at 4°C/min.

Inducibility of esterases/cutinases. Inducibility of the esterases/cutinases was determined by inoculating duplicate culture tubes (20 × 150 mm) containing TYE or GA ± apple cutin (5 ml/tube) as above and shaking at room temperature for 9 days. Samples were removed at days 3, 6, and 9 of incubation, and esterase activity was determined as stated above. Each strain was tested in two to three experiments.

Results

Esterase production. The ability to cleave the ester bond present in the colorless substrate *p*-nitrophenyl butyrate (PNB) is a simple, rapid, presumptive test for microbial cutinase activity [18]. All known cutinases, with one recently reported exception [3], can cleave this bond, releasing *p*-nitrophenol, a yellow-colored product that absorbs at 405–410 nm.

Each *Streptomyces* strain was examined for esterase production in the two broth media. The first, GA broth, was amended with tomato cutin (GA-T) (for *S. ipomoea*) or apple cutin (GA-GDA) (for all other strains). All strains exhibited limited to no visible growth in this medium. Only three strains consistently demonstrated more than 100 nmol/min/ml of activity in the culture fluid over the 14-day incubation period; *S. "scabies"* ATCC 15485 (maximal activity of 511 nmol/min/ml) and IMRU 3018 (172 nmol/min/ml), and *S. acidiscabies* ATCC 49003 (247 nmol/min/ml).

Much improved growth occurred for the *Streptomyces* spp., except for *S. ipomoea*, in TYE broth amended with apple cutin (TYE-GDA). *S. ipomoea* strains were also tested in TYE-GDA supplemented with 2% (wt/vol) mannitol. Addition of mannitol led to luxuriant growth of these strains. Only *S. acidiscabies* ATCC 49003 (maximal activity of 262 nmol/min/ml); *S. "scabies"* B1515 (138 nmol/min/ml), B391 (153 nmol/min/ml), B3262 (149 nmol/min/ml) and IMRU 3018 (869 nmol/min/ml); *S. badius* ATCC 19888 (160 nmol/min/ml) and *S. viridisporus* ATCC 27479 (624 nmol/min/ml) consistently reached esterase values over 100 nmol/min/ml in these media over the 14-day incubation period.

Confirmation of cutinase activity. Culture fluids from *S. "scabies"* ATCC 15485 and *S. scabies* ATCC 49173 grown in GA broth, as well as culture fluids from *S. acidiscabies* ATCC 49003, *S. scabies* ATCC 49173, all *S. "scabies"* strains, *S. badius* ATCC 19888, and *S. viridisporus* ATCC 27479 grown in TYE-GDA, were examined for cutinase activity. Culture fluids were incubated with purified cutin powder for 18 h, and then the released cutin monomers were extracted and analyzed by HPLC. Highest cutinase activity was present in culture fluids of *S. acidiscabies* ATCC 49003, *S. "scabies"* IMRU 3018, and *S. badius* ATCC 19888 grown in TYE plus apple cutin, and in culture fluids of *S. "scabies"* ATCC 15485 grown in GA plus apple cutin (Table 1). Representative chromatograms of base-hydrolyzed apple cutin [7] and of apple cutin treated with culture fluids exhibiting cutinase activity are shown in Fig. 1. The identity of the released cutin monomers was confirmed by GC/MS analysis (Fig. 2). Culture fluids of all four strains caused release of the major cutin monomers reported for GDA cutin from fruits [9]. Addition of the nonionic detergent *n*-octylglucoside to the assay mixtures resulted in complete inhibition of cutinase activity for all four strains.

Enzyme induction. Esterase/cutinase activity of the four confirmed cutinase-producing *Streptomyces* strains was induced by addition of cutin to the medium, as determined with the PNB assay. The data for one representative experiment are shown in Table 2. Highest induction was exhibited by *S. "scabies"* IMRU 3018 (maximum of 14- to 17-fold), while lowest induction was for *S. acidiscabies* ATCC 49003 (maximum of 2- to 3-fold).

Discussion

The ability to produce cutinase does not appear to be a trait common among *Streptomyces* spp. that

Table 1. Esterase and cutinase activity of *Streptomyces* culture fluids

Bacterium	Strain	Medium ^a	Esterase ^b (nmol/min/ml)	Released cutin monomer ^c (mg/18 h/ml culture fluid)
<i>S. acidiscabies</i>	ATCC 49003	TYE	220	1.38
<i>S. scabies</i>	ATCC 49173	GA	0	0.03
		TYE	11	0
<i>S. "scabies"</i>	ATCC 3352	TYE	83	0.20
	ATCC 15485	GA	500	0.72
	IMRU 3018	TYE	850	2.30
	IMRU 3647	TYE	149	0.60
	B391	TYE	124	0
	B1515	TYE	108	0.1
	B2799	TYE	97	0.3
	All others	TYE	<100	<0.25
<i>S. badius</i>	ATCC 19888	TYE	160	2.30
<i>S. viridisporus</i>	ATCC 27479	TYE	624	0

^a Broth media supplemented with 0.4% apple cutin. TYE, tryptone-yeast extract; GA, glycerol-asparagine.

^b Determined by PNB assay as described in Materials and Methods.

^c Culture fluids were incubated with purified cutin powder for 18 h, and then the released cutin monomers were extracted and analyzed by HPLC. Total monomer released was based on reference to a standard curve of area units per unit weight prepared for 9,10,18-trihydroxyoctadecanoic acid [7].

have the ability to cause disease on potato or sweet potato. We have identified, using a novel HPLC technique developed in our laboratory, four cutinase-producing strains of *Streptomyces*: the plant pathogens *S. acidiscabies* ATCC 49003 (type strain), *S. "scabies"* IMRU 3018, and ATCC 15485, and the non-plant pathogen *S. badius* ATCC 19888. Only a single strain of *Streptomyces* was previously reported to be capable of cutin degradation, and this strain was identified as *S. scabies* [14]. This cutinase-producing strain may be reclassified under the newly revived taxonomic description [12].

Streptomyces acidiscabies is a recently described bacterial species for acid-tolerant strains of *Streptomyces* pathogenic towards potato [13]. In contrast to *S. scabies*, this bacterium is able to grow on agar medium at pH 4.0 and can cause scab disease on potatoes in soils with pH values below 5.2 [13]. *Streptomyces "scabies"* strain IMRU 3018 (= ISP 5078) was described as the neotype strain for *S. scabies*, but this strain, as well as *S. "scabies"* strain ATCC 15485, do not fit the revived description of *scabies* and have been designated as *S. albidoflavus* [8, 12]. Two primary reasons that these two strains are not considered *S. scabies* are that they do not produce the pigment melanin and they have a rectiflexuous rather than spiral spore chain morphology. Interestingly, these two strains are very similar to each other in morphological and physiological characteristics [12]. Other strains included in our study that were reported not to be *S. scabies* under the

revived description and have been designated as *S. albidoflavus* were *S. "scabies"* ATCC 10246, ATCC 3352, and IMRU 3029 [8, 12, 21].

Streptomyces viridisporus strain ATCC 27479 exhibited high extracellular esterase activity, but the esterase was incapable of cutin hydrolysis. This esterase may be similar to extracellular esterases produced by two pathogenic strains of *S. scabies* [15]. The *S. scabies* esterases hydrolyze *p*-nitrophenyl butyrate and are inducible by zinc. The esterase produced by one strain was purified and characterized [15]. This esterase differs in several respects from the previously reported esterase/cutinase of *S. scabies* [14]. No release of fatty acid monomers from either apple cutin or potato suberin was detected after incubation with the purified *S. scabies* esterase [15].

Production of cutinase by *S. "scabies"* strain ATCC 15485 was higher in GA broth amended with cutin, which supported only limited growth, than in TYE broth, which supported much more luxuriant growth. Lin and Kolattukudy [14] reported cutinase production by *S. scabies* in a basal mineral medium with cutin as the sole source of carbon, even though bacterial growth was very limited. In contrast, *S. "scabies"* strain IMRU 3018 and *S. badius* ATCC 19888 produced higher levels of cutinase in TYE broth, and *S. acidiscabies* produced equivalent amounts in both media.

Because of the apparent rarity of the ability to produce esterases that hydrolyze cutin and presum-

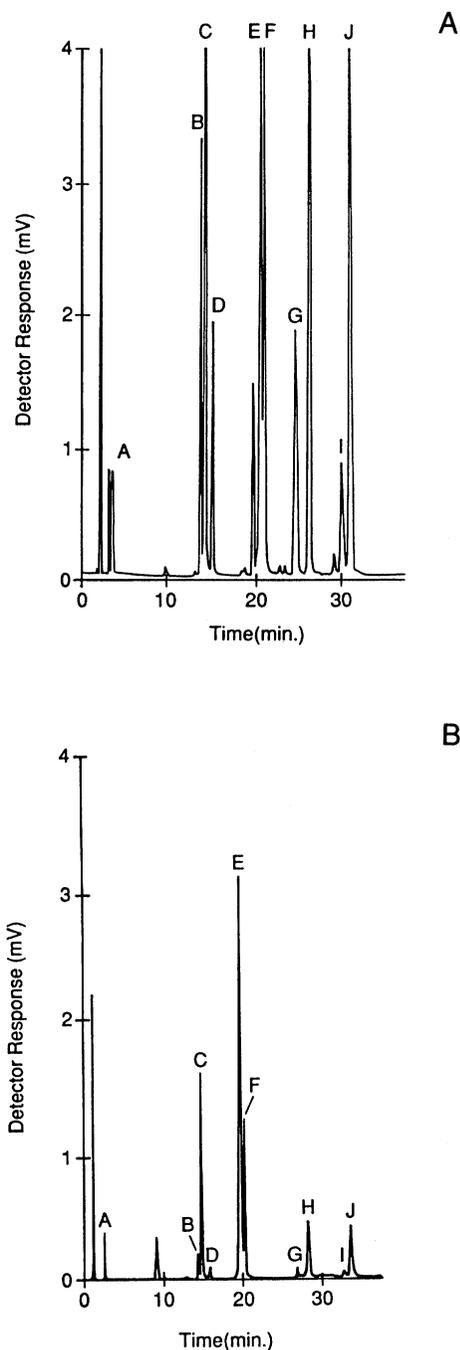


Fig. 1. Comparison of HPLC chromatograms of monomers released from apple cv. Golden Delicious fruit cutin treated with either acidified methanolic KOH (A) or culture fluid of *Streptomyces badius* ATCC 19888 grown in tryptone-yeast extract broth supplemented with apple cutin (B). Identity of cutin monomers: A, hexadecanoic + octadecanoic acids; B, 18-hydroxyoctadeca-9,12-dienoic acid; C, 18-hydroxyoctadeca-9-enoic acid; D, 16-hydroxyhexadecanoic acid; E and F, 9,10-epoxy-18-hydroxyoctadecanoic acid and 9,10-epoxy-18-hydroxyoctadeca-12-enoic acid; G, 9,18-dihydroxyoctadecanoic acid; H, 10,18-dihydroxyoctadecanoic acid; I, 9,10,18-trihydroxyoctadeca-12-enoic acid; J, 9,10,18-trihydroxyoctadecanoic acid.

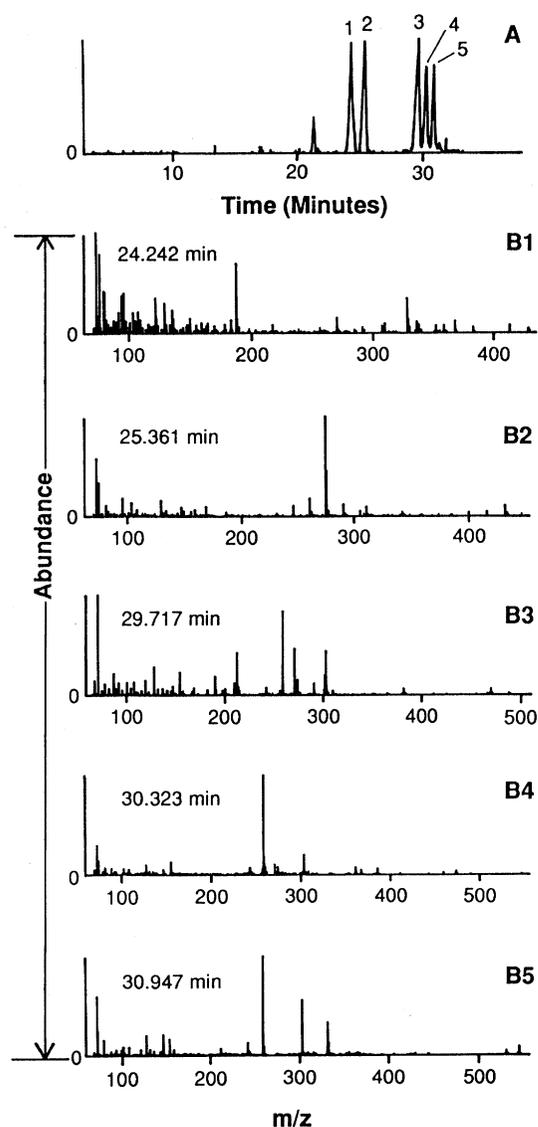


Fig. 2. GC profile (A) and GC/MS (B) of methylated cutin monomers released from apple cv. Golden Delicious fruit cutin after incubation with culture fluid of *Streptomyces* "scabies" strain ATCC 15485. Identity of cutin monomers: (1) 18-hydroxyoctadeca-9,12-dienoic acid; (2) 10,16-dihydroxyhexadecanoic acid; (3) methoxyhydrins derived from 9,10-epoxy-18-hydroxyoctadeca-12-enoic acid; (4) methoxyhydrins derived from 9,10-epoxy-18-hydroxyoctadecanoic acid; (5) 9,10,18-trihydroxyoctadecanoic acid.

ably suberin [5], such esterases may not be required for pathogenicity of *S. scabies* or *S. ipomoea*. *S. scabies* normally enters tubers through lenticils [10], which may or may not contain suberized cell walls [4]. However, the ability to produce such esterases may be more widespread than indicated by this study. The utilization of additional sources of cutin,

Table 2. Induction of esterase/cutinase by cutin

Bacterium	Strain	Medium	Esterase activity (nmoles/min/ml) ^a					
			Day 3 cutin		Day 6 cutin		Day 9 cutin	
			(+)	(-)	(+)	(-)	(+)	(-)
<i>S. acidiscabies</i>	ATCC 49003	TYE	87 ± 53 ^b	64 ± 20	103 ± 8	67 ± 25	81 ± 27	70 ± 42
<i>S. "scabies"</i>	ATCC 15485	GA	213 ± 53	28 ± 13	370 ± 42	42 ± 6	225 ± 106	47 ± 4
	IMRU 3018	TYE	169 ± 62	56 ± 9	346 ± 146	24 ± 8	119 ± 44	12 ± 0
<i>S. badius</i>	ATCC 19888	TYE	36 ± 8	9 ± 2	145 ± 64	42 ± 14	287 ± 11	34 ± 1

^a Bacteria were grown in broth media supplemented with purified, powdered cutin. Samples of culture fluids were removed periodically, and esterase activity was determined as stated in Materials and Methods.

^b Values given are averages from two replicate cultures per strain ± standard deviation.

use of suberin, or additional changes in media and culture conditions may be required for enzyme induction by other *Streptomyces* strains. Production of enzymes that can degrade suberin may facilitate bacterial growth and penetration through young, differentiating, natural periderm, lenticils with suberized cell walls, or developing wound periderm layers containing partially suberized cell walls.

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