0273-2289/93/4202-03—0161$04.80

Reviews in Biotechnology

Isolation Properties and Potential Applications of Thermophilic Actinomycetes

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ABSTRACT

Actinomycetes comprise a large and diverse group of largely mycelial bacteria, many of which are important ecologically and are exploited commercially for the production of natural products such as antibiotics and enzymes. Thermophilic species are also known but these are relatively poorly studied compared with the predominant mesophilic genera. This article describes habitats and methods for isolation and recovery of thermophilic actinomycetes, such as *Streptomyces, Thermomonospora,* and *Thermoactinomyces.* General properties are discussed with respect to mesophilic species and their potential for the production of enzymes concerned with hydrolysis of complex macromolecules described. Their ability to synthesize antibiotics is reviewed and the advantages of production at high temperatures illustrated with reference to granaticin production by *Streptomyces thermoviolaceus.* Work with some of the better studied thermophilic species indicates the presence of novel properties. More intensive investigations of their properties, as well as those of actinomycetes that grow at extremes of pH or salinity, may lead to the discovery of novel natural products.

Index Entries: Thermophiles; thermophilic actinomycetes; ecology; isolation; enzymes; antibiotics.

INTRODUCTION

Actinomycetes comprise a large diverse group of gram-positive predominantly mycelial bacteria that inhabit a range of environments. Some are pathogenic, but the majority are saprophytes in soil, water, and plant material where they play important roles in soil structure and composting (*1,2*). They secrete extracellular enzymes that are important in the breakdown of complex organic materials, such as proteins, nucleic acids, polysaccharides, and plant polymers in natural environments, and are therefore important for cycling of carbon in the biosphere. Many actinomycetes also produce bioactive natural products of commercial importance. Preeminent in this respect are *Streptomyces* spp., which are major producers of antibiotics (*3*). Because mesophilic species have been extensively isolated and characterized, the search for novel products has switched in emphasis to rarer genera of actinomycetes or to well-characterized ones that are found in unusual environments. The rationale for these approaches is that they may produce novel metabolities. One such group, the topic of this article, comprises the thermophilic actinomycetes. Thermophilic bacteria have become the focus of much interest with respect to their biotechnological potential. Three groupings based on temperature ranges for growth have been proposed (*4*): thermophiles, which have a minimum growth temperature (T_{min}) no lower than 30°C an optimum (T_{opt}) of around 50°C and a maximum temperature (T_{max}) for growth above 60°C; extreme thermophiles (T_{min}, 40°C; T_{opt}, 65°C, and T_{max}, 70°C); and hyperthermophiles or ultrathermophiles (T_{min}, not defined; T_{opt}, 100°C, and T_{max} currently around 115–120°C). Thermophilic species of two genera of aerobic eubacteria have been more extensively studied than any others; these are *Thermus* (*T. aquaticus*) and *Bacillus* (*B. stearothermophilus*). However, as indicated by Bergquist and Morgan (*5*), thermophiles have yet to replace mesophilic counterparts in major biotechnological processes, although specialized applications exist as exemplified by the commercial success of *Taq* polymerase used in the polymerase chain reaction (PCR) for the amplification of DNA. The thermophilic actinomycetes, with a few exceptions, thrive best at temperatures between 45 and 60°C (*6*) and, therefore, fall into the lower end of the thermophilic grouping. However, in the context of mesophilic actinomycetes, they can be viewed as true thermophile representatives within the order Actinomycetales. Here, methods for their recovery and isolation will be reviewed, and their potential in biotechnological processes illustrated.

HABITATS

Actinomycetes are well adapted to growth on solid substrates and are ubiquitous in natural environments because of their metabolic diversity and evolution of specific mechanisms for dispersal (*7*). There are many

environments that reach temperatures of 40–70°C; these include solar-heated soils, sites of hot industrial discharges, coal mining refuse tips, and decaying plant matter of which composting is a good example. The latter is probably a favored site for screening for the presence of thermophilic actinomycetes. The material being composted provides a source of nutrients, moisture, and microbial inoculum. Preparation of mushroom compost is a good example of a commercial process in which thermophilic bacteria play a key role. It is an aerobic, solid substrate (horse bedding, chicken manure) fermentation mediated by a diverse, active, highly competitive, largely gram-positive bacterial microflora of which the thermophilic actinomycetes comprise an important component (*6*). Goodfellow et al. (*8*) isolated 50 thermophilic streptomycetes from garden and mushroom compost. More recently, Gadkari et al. (*9*) isolated an unusual thermophilic streptomycete from soil covering a burning charcoal pile, and a novel acid-tolerant *Thermoactinomycetes* has been isolated from a low-pH pine forest soil (C. Yallop, S. T. Williams, and C. Edwards, unpublished work).

ISOLATION

Recovery and isolation of thermophilic actinomycetes is best at 50°C; a number of species grow poorly at temperatures of 55°C and greater (*7*). Isolation of thermophilic actinomycetes from mushroom compost illustrates some of the problems in assessing their true diversity (Table 1). The use of traditional microbiological techniques involving dilution plating is hampered by the tendency of more rapidly growing thermophilic bacteria, mainly *Bacillus*, to rapidly colonize isolation plates and severely limit the detection of the more slowly growing actinomycetes. As is apparent from Table 1, the bacilli are present at much higher cell densities in compost (ca. 10^{-10} g compost^{-1}), and some species have the tendency to produce extracellular polysaccharides, which result in confluent growth of *Bacillus* on isolation plates further hampering the growth of thermophilic actinomycetes. Methods that involve selective media to reduce the growth of *Bacillus*, but allow growth of actinomycetes have been developed (*10*), and some of these are shown in Table 1 for two self-heating substrates, compost and hay. These methods involve selective media that restrict the growth of bacilli and allow improved recovery of the actinomycete population. This contains brown colonies of *Thermomonospora chromogena* and white thermomonosporas (kanamycin), *Thermoactinomyces* (novobiocin), and various species of thermophilic streptomycetes (thiostrepton). Changing the nutrient formulation of the agar plates can also restrict growth of *Bacillus* and improve recovery of actinomycetes; e.g., R5-agar, usually used to regenerate streptomycete protoplasts, improves the recovery of streptomycetes and allows detection of *Saccharomonospora viridis*. Also

Table 1
Selective Isolation of Thermophilic Actinomycetes from Natural Substrates (Taken from *2,10*)[a]

Selection condition	Actinomycete	A		B	
		Actinomycete	Bacilli	Actinomycete	Bacilli
Mushroom Compost					
None	Total	10^7	10^{10}	1.5×10^3	Confluent growth
Kanamycin	*T. chromogena*	10^6	10^8	412	9
(25 μg mL^{-1})	white thermomonosporas	10^6	10^8	0	9
Novobiocin	*Thermoactinomyces*	10^8	10^8	68	23
(25 μg mL^{-1})					
R5	*Saccharomonospora viridis*	10^4	10^7	2	2
	Streptomycetes	10^7	10^7	242	2
Thiostrepton	Streptomycetes	10^5	10^6	25	1
(50 μg mL^{-1})					
Hay					
None	Total	NT*	NT	1	NT
R5	*S. viridis*	NT*	NT	874	NT
	Faenia rectivirgula	NT*	NT	154	NT
R8	*S. viridis*	NT*	NT	1,121	NT
	F. rectivirgula	NT*	NT	220	NT

[a] Colony forming units (cfu g compost^{-1}) after dilution plating (A) or recovered per stack of Anderson sampler plates (B).
NT* = not tested.

shown in Table 1 are bacterial numbers obtained using an Andersen sampler. This involves drying compost samples, a treatment that kills vegetative cells but allows survival of spores, and agitation in a sedimentation chamber to produce a cloud of spores that is then drawn from the chamber as an air stream that is passed through an Andersen sampler stack. Each isolation plate in the stack is separated by a metal disk; these have graduated hole sizes such that the disk bearing the smallest holes is at the bottom of the stack where actinomycete spores are generally recovered. *Bacillus* spores tend to clump together or adhere to the dried compost and are generally not maintained in air suspension. The success of the method is illustrated in Table 1, and shows that for hay samples two new thermophilic species (*Faenia rectivirgula* and *Saccharomonospora viridis*), normally present at low numbers or remaining undetected, were recovered at high numbers. By using the appropriate selective media, the *Bacillus* population can be reduced to very low numbers.

GENERAL PROPERTIES

Temperature ranges for growth of thermophilic actinomycetes seldom exceed a maximum temperature of 60°C. Thermophilic actinomycetes that are monosporic (produce a single spore) include *Thermomonospora Thermoactinomyces* and *Saccharomonospora*, and these grow well in the range of 30 to 55°C, although *Thermoactinomyces* may exceed this and grow up to 65–70°C. Thermophilic thermoactinomycetes produce endospore-like spores, as well as a variety of natural products. The properties and taxonomy of the monosporic genera have been presented by McCarthy and Cross (*11*). They concluded that the genus *Thermomonospora* divided into two distinct clusters, one of which comprised *T. chromogena*, which they proposed should be assigned a new genus, and the other consisted of the white thermomonosporas (*T. fusca, T. curvata*, and *T. alba*). Saccharomonosporas showed no growth at 60°C, but all isolates could grow at 55°C. Thermophilic streptomycetes only grow up to a temperature of 58°C, except for the recently isolated *S. thermoautotrophicus*, which is able to grow up to 65°C and shows growth-associated activities at 70°C (*9*). A taxonomic study of 50 thermophilic streptomycete isolates showed that they formed distinct centers of variation when compared to 201 mesophilic species. It was concluded that the thermophilic strains were not variants of established mesophilic taxa. General properties that distinguished them included no melanin production, and seldom showing activity against a number of compounds, including chitin, guanine, urea, and failure to grow in the presence of 7% NaCl. Few strains produced pigmented colonies or diffusible pigments, but most of the isolates studied produced protease, lipase, and amylase activities, as well as utilizing a wide range of compounds as sole carbon sources (*8*).

Bell et al. (*12*) described an unusual thermophilic streptomycete called *Streptomyces* G26, which was isolated from compost. This proved to be a facultative autotroph capable of aerobic growth on CO or CO_2/H_2. Soluble extracts of the streptomycete were shown to contain enzymes of the Calvin cycle together with a unique CO oxidoreductase that had a very low apparent K_m for CO, which was comparable with the CO oxidoreductase of *Pseudomonas thermocarboxydovorans*. Activities of the enzyme were highest at 60–65°C. Like all known carboxydotrophic bacteria at this time, *Streptomyces* G26 could also grow on organic growth substrates. These results provide an impetus for seeking unusual actinomycetes and screening unusual habitats, especially since Bell et al. (*12*) reported that *Streptomyces* G26 was representative of more than 100 strains of CO-utilizing actinomycetes isolated by them from soils and compost. More recently, Gadkari et al. (*9*) have isolated and partially characterized a thermophilic CO- and H_2-oxidizing obligate chemolithotrophic actinomycete from burning charcoal piles. They named the organism *Streptomyces thermoautotrophicus* on the basis of G+C content, phospholipid profile, and other chemotaxonomic and morphological properties. It grew on CO (doubling time 8 h), H_2 plus CO_2 ($g = 6$ h), and other CO-containing gases, such as car exhaust. It was incapable of growth on any of the organic media tested. Chemoautotrophic growth was optimal at 65°C with no growth occurring below 40°C, although CO-grown cells could oxidize CO in the temperature range of 10 to 70°C. Analysis of the genes encoding subunits of CO dehydrogenase has shown that they are conserved in all the carboxydotrophic bacteria examined with the exception of *S. thermoautotrophicus* (*13*). This streptomycete has proven to be a unique carboxydotroph with respect to its apparent inability to utilize organic substrates. It represents an extremely unusual actinomycete and is the most thermophilic streptomycete isolated to date. *Streptomyces thermoautotrophicus* has recently been shown to utilise N_2 as the sole nitrogen source when grown chemolithotrophically with CO or H_2 plus CO_2 under aerobic conditions at 65°C. It is likely to contain an unusual nitrogen-fixing system because preliminary results have shown that it cannot reduce acetylene, as is the case for other diazotrophic bacteria studied thus far; nitrogen fixation is resistant to inhibition by acetylene or ethylene, CO, or H_2; under stringent conditions, *nif*H and *nif*DK gene probes from *Klebsiella pneumoniae* failed to hybridize with *S. thermoautotrophicus* total DNA (*14*). The discovery of such a novel organism with unusual metabolic activities augurs well for the discovery of further unusual actinomycetes with the attendant possibility for the biosynthesis of novel natural products.

ENZYMES

Enzymes from thermophilic bacteria have become of intense interest, particularly for their potential as biocatalysts in biotechnology. Recent advances in molecular genetic methods have heralded the possibility of

endowing increased thermostability through protein engineering. It is now well understood that small changes in the right regions of a protein are sufficient to alter its thermostability markedly. These can be as small as a single amino acid change. There is also a general relationship between enzyme thermostability and the thermophilicity of the host bacterium (4); that is, enzymes become increasingly thermostable as one moves up the temperature scale through psychrophiles, mesophiles, thermophiles, and ultrathermophiles. This is of some significance to those bacteria, such as the actinomycetes, that are involved in biodegradation of polymeric substrates via extracellular hydrolases. The enzymes must be sufficiently thermotolerant to be stable under the prevailing temperatures, which are often prone to fluctuate. As a result, such enzymes may exhibit better physical and chemical stability, which makes them targets worthy of investigation. However, at this point, we should sound a note of caution. Thermophilic enzymes need not have more rapid catalytic rates than their mesophilic counterparts. For a given enzyme, it would appear that thermophilic versions have the same catalytic efficiency and molecular flexibility as mesophilic ones at their respective temperature optima. It follows that a thermophilic protein will have reduced molecular flexibility under mesophilic conditions. The advantages to more efficient catalysis at high temperatures would appear to lie at the substrate level rather than the enzyme. For example, proteins may be denatured and therefore more amenable to attack by proteases; lipids will be predominantly in the liquid phase and therefore more readily broken down by lipases (4,5).

Table 2 describes some of the enzymes of thermophilic actinomycetes that have been studied together with an indication of their heat tolerance. Because high temperatures are often used for the hydrolysis of starch in industry, enzymes from thermophilic bacteria have been sought. α-Amylase is widely distributed among the streptomycetes, the enzyme from the mesophile *Streptomyces hygroscopicus* is used industrially to hydrolyze starch during the production of high-maltose syrups, and the gene for the amylase has been cloned and sequenced (*30*). Bahri and Ward (*17*) have cloned the α-amylase from *S. thermoviolaceus* in *Escherichia coli* and *S. lividans*. This is a common approach when cloning genes from thermophilic organisms. Expression within a mesophilic background allows the study of the properties of the cloned protein, and facilitates its purification by inactivating and precipitating the host proteins using temperatures at which the thermophilic protein is stable (*31*). In *E. coli* and *S. lividans*, the α-amylase from *S. thermoviolaceus* had the same molecular mass and properties as in the parent strain. The cloned protein activity was stabilized at 70°C by calcium (*17*). Goldberg and Edwards (*16*) purified and characterized a single amylase from *S. thermoviolaceus* subsp. *apingens*, a nonpigmented (and therefore nonantibiotic-producing) variant of *S. thermoviolaceus*. Its properties were generally similar to those of the enzyme from the pigmented strain described by Bahri and Ward (*17*) and also resembled the commercially used amylase from *S. hygroscopicus* (*30*).

Table 2
Some Enzymes and Their Properties from Thermophilic Actinomycetes

Enzymes	Actinomycete	Comments	References
α Amylase	*Thermoactinomyces vulgaris*	$t_{1/2}$ 60°C—2 h, stabilized by calcium	*15*
	Streptomyces thermoviolaceus	$t_{1/2}$ 60°C—10 min; increased to 55 min with 5 m*M* Ca^{2+}	*16*
		α-Amylase gene cloned into *E. coli* and *S. lividans*; stable for 22 h in the presence of Ca^{2+} at 50°C	*17*
	Thermomonospora curvata	$t_{1/2}$ 70°C—60 min	*18*
	Thermomonospora sp.	$t_{1/2}$ 65°C—4 h	*19*
Lipase	*S. thermoviolaceus*	$t_{1/2}$ 70°C—70 min, no stabilization by calcium	C. Edwards, unpublished work
Protease	*Thermoactinomyces vulgaris*	$t_{1/2}$ 70°C—10 min, increased to 30 min with Ca^{2+}	*20*
	Thermoactinomyces sp.	Carboxypeptidase, 40% activity after 2 h at 60°C	*21*
	S. thermoviolaceus	Multiple proteases identified: $t_{1/2}$ 70°C of metalloprotease—80 min	*22*

Xylanase	*Thermomonospora fusca*	Crude activity $t_{1/2}$ 70°C—6 h; further purification revealed six proteins with xylanase activity	*23*
	Thm.fusca KW3	Xylanase produced maximally at low growth rates in continuous culture	*24*
	Saccharomonospora viridis	$t_{1/2}$ 70° approx 60 min	*25*
		Enzyme used to reduce xylan content of birchwood pulp, which was subsequently used to make modified paper	*26*
	S. thermoviolaceus	Two different enzymes purified and partial amino acid sequence determined; one enzyme had temperature optimum for activity of 70°C, and the other 60°C	*27*
	Thermomonospora curvata	Three different endoxylanases identified with $t_{1/2}$ 75°C of 21, 151, and 302 h, respectively	*28*
β-xylosidase	*Thermomonospora fusca*	$t_{1/2}$ 70°C—1.5 h; trimeric enzyme	*29*

Proteases account for approx 55% of the worldwide enzyme market (32), and they have a wide range of applications in the detergents, brewing, baking, leather, and dairy industries, as well as in medicine. The inherent stability of such enzymes from thermophilic bacteria has made them attractive targets for mediating processes in these industries. Extracellular proteases are probably the most widespread of all microbially secreted enzymes (33), and there are several reports on their production by mesophilic *Streptomyces*, but only a few from thermophilic strains. Generally proteases in actinomycetes tend to be produced during the stationary phases of growth, for example, the heat-stable protease produced by *Thermomonospora fusca* (34). A metallo-carboxypeptidase from *Thermoactinomyces* sp. was stabilized at high temperature by calcium ions (21). Calcium has been implicated in stabilization of a number of thermophilic enzymes. Presumably this occurs through specific or nonspecific binding sites that allow for additional bonding and, hence, increased thermostability within the protein molecule (31). *Streptomyces thermoviolaceus* produced maximum protease activity at 50°C. A purification method based on acetone precipitation also allowed concentration and characterization of activity. The presence of multiple proteases was suggested on the basis of inhibitor studies and degradation of fluorogenic peptide substrates. These were predominantly serine- and metallo-type enzymes (22), and extracellular activity could be detected during the early growth phase concomitant with the efflux of antibiotic from the mycelium. At least four different proteases have now been identified in this streptomycete, and these exhibit different molecular sizes as well as a varied thermal tolerance (35).

By far the greatest interest has centered on the capacity of the actinomycetes, and the thermophilic species in particular, to degrade plant-derived materials, especially lignocellulose. This is hardly surprising in view of the major role they are known to have in biodegradation and turnover of polymeric materials in natural environments, as well as their growth as branching and invasive hyphae, which penetrate solid substrates ensuring more rapid degradation. Lignocellulose is made up of lignin, cellulose, and xylan, and comprises an important renewable energy source that, providing the right organisms are used, may be exploited for bioconversion, which may be of commercial benefit. Two general areas of exploitation can be envisaged as a result of lignocellulose degradation: production of biomass that can be used as a source of cheap single-cell protein or for mediating a secondary process; and release of monomers from polymeric plant materials that can be used by a second organism for the growth and production of commercial biochemicals. The advances in development and application of molecular genetic techniques will no doubt allow considerable progress and realization of the above aims. Lignin is particularly recalcitrant to biological breakdown, and therefore, cellulose would appear to be the major polymer available for biodegradation. However, xylan,

which ranks second in abundance and which may comprise up to one-third of the total dry weight in higher plants, also comprises a significant renewable resource available for biotechnological applications (*28,36*). Lignocellulose-degrading actinomycetes have been well reviewed by McCarthy (*36*). Some of the key enzymes that have been studied in thermophilic actinomycetes are shown in Table 2.

A number of general points can be made from studies of cellulose and xylan degradation by thermophilic actinomycetes. Multiple forms of enzymes involved in breakdown of polymeric materials appear to be common. For example, up to six different xylanases were identified in *T. fusca* on the basis of zymogram analysis (*23*). Similarly, Stutzenberger and Bodine (*28*) showed the presence of three endoxylanases in *T. curvata*. A variable, but often high degree of thermotolerance is often found in these enzymes, which have multiple forms, and may reflect the environment of thermophilic actinomycetes, which commonly inhabit areas of fluctuating temperatures. Multiple enzymes that display different properties may be necessary to ensure continued biodegradative activity in such habitats. For example, β-xylosidase from *T. fusca* had a $t_{1/2}$ at 65°C of 8 h (*29*); three endoxylanases in *T. curvata* displayed $t_{1/2}$ at 75°C of 21, 151, and 302 h, respectively (*28*). An interesting observation by Bachmann and McCarthy (*23*) revealed that two or more enzymes present together had more degradative activity than the sum of their individual activities. This synergistic phenomenon was measured during hydrolysis of straw by α-arabinofuranosidase acting in concert with endoxylanase. It is tempting to infer that synergistic degradation of polymeric substrates must occur in natural environments. Some evidence for this comes from recent work on the thermophile *T. curvata*. When this actinomycete was grown on polymeric substrates, such as xylan protuberances, of the cell surface could be seen in electron micrographs, these were sparse or nondetectable when the organism was grown on simpler substrates. The surface protuberances were associated with an increase in cell-bound exoenzymes, suggesting the formation of complexes of different and multiple forms of hydrolytic enzymes (*37*). Similar cell-surface blebbing has been detected in scanning electron micrographs of *S. thermoviolaceus*, and these are most pronounced at the time of antibiotic and protein secretion from the mycelium (C. Edwards, unpublished work). Rothlisberger et al. (*24*) recently showed that growth rate had a pronounced effect on the activity of an extracellular thermostable endoxylanase of *T. fusca* grown in continuous culture. Growth rates as high as 0.23 h^{-1} (doubling time 3 h) could be achieved, although xylanase activity was greatest (2.5 U mL^{-1}) at low growth rates (0.03 h^{-1}) and fell to approx 0.5 U mL^{-1} at $\mu = 0.1$ h^{-1}. Similar experiments in *S. thermoviolaceus* also revealed that extracellular protease activities were greatest at low growth rates in carbon-limited continuous cultures. Tsujibo et al. (*27*) have recently purified two endoxylanases from *S. thermoviolaceus*,

and have shown that one has a high amino acid sequence homology with exoglucanase from *Cellulomonas* sp. and the other with a xylanase from *Bacillus pumilus*.

ANTIBIOTIC PRODUCTION

There are relatively few reports concerning antibiotic production by thermophilic actinomycetes. Many also tend to be dated as can be seen from Table 3. Thermorubin is one of the few that has been subjected to investigation, particularly with respect to its chemical structure in relation to mode of action, and also for the synthesis and testing of chemical derivatives (*40*). Granaticin synthesis in *S. thermoviolaceus* has been studied with respect to growth physiology. This antibiotic is also produced by many mesophilic streptomycetes, and is synthesized via the polyketide pathway by mixed biogenesis of eight acetate units and one dideoxyglucose moiety (*48,49*). The antibiotic effect of granaticin is by inhibition of RNA synthesis of gram-positive bacteria, notably *Bacillus* sp. (*46,50*). Although granaticin has no commercial significance, it has a number of properties that make it a useful model system for investigating antibiotic synthesis in a thermophilic streptomycete. It is the product of a major antibiotic biosynthetic pathway (polyketide pathway) by which many commercially important antibiotics are synthesized. It is a pigmented pH-sensitive molecule (red in acid; blue in alkali) and can therefore be easily quantified by spectrophotometry.

Early work on *S. thermoviolaceus* showed that a wide variety of carbon sources could support growth and antibiotic synthesis, control of culture pH was essential for maximizing the yield of granaticin, and the best carbon source for granaticin production was proline (*51*). More detailed analysis of the effects of temperature on growth and antibiotic production revealed that 45°C appeared to be the optimum. Growth was most rapid at 50°C with a doubling time of 1 h. At 45°C growth appeared biphasic with the onset of detectable extracellular antibiotic coinciding with the inflexion point of the growth curve and continuing during the second, slower growth phase (*47*). These data showed that for *S. thermoviolaceus*, secondary metabolism commenced relatively early at a time when all the major nutrients were still in excess. It also appeared that growth rate was more important as a determinant for antibiotic production. Further work established a possible reason for the biphasic growth of the streptomycete. Large amounts of protein were secreted at the same time as antibiotic efflux from the mycelium, and by the end of growth, the extracellular protein comprised 50% of the dry weight. Since growth in mycelial organisms is best monitored by increase in dry weight, loss of mass owing to protein secretion or lysis would help to explain the slower accretion of biomass during the secondary growth phase. Protease activity was identified as a component and marker for the timing of protein secretion (*22*).

Table 3
Some Antibiotics Produced by Thermophilic Actinomycetes

Antibiotics	Actinomycetes	Comments	Reference
Thermomycin	*Streptomyces thermophilus*	Bacteriostatic against *Corynebacterium diphtheriae,* optimal production—4 d at 60°C	*38*
Thermorubin	*Thermoactinomyces antibioticus*	Bactericidal in vitro and in vivo	*39*
		Binds 1:1 with *Escherichia coli* 70S ribosomes and 50S and 30S subunits inhibits protein synthesis; produced at 50°C	*40*
T-SA-125	*Thermomonospora* sp.		*41*
Thermoviridin	*Thermoactinomyces viridis*	Most active against gram+ve bacteria; produced at 45°C	*42*
Thermothiocin	*Thermoactinopolyspora cormialis*	Bactericidal in vivo and in vitro; sulfur-containing polypeptide	*43*
Granaticin	*Streptomyces thermoviolaceus*	Active against some gram+ve bacteria	*44*
		Also produced by many mesophilic species	*45*
		Thought to inhibit RNA polymerase in *Bacillus subtilis*	*46*
		Produced up to 55°C in *S. thermoviolaceus*	*47*

Because of the rapid doubling times observed during the first phase of growth and because secondary metabolites are synthesized during active growth, it was possible to study the effects of growth rate on growth physiology of *S. thermoviolaceus* using nutrient-limited chemostats. These experiments allowed a fuller assessment of the effects of temperature on granaticin synthesis, secretion of extracellular protein, and amount of protease activity as components of secondary metabolism. Cultures were grown in a simple salts medium with glutamate limitation at 37, 45, and 50°C. Both temperature and growth rate were found to affect the amount of each metabolite profoundly. Granaticin yields were highest at growth rates of 0.1–0.15 h^{-1} at 37°C, 0.175 h^{-1} at 45°C, and 0.045 h^{-1} at 50°C. Protease activities were greatest at low growth rates and fell with increased dilution rates. Measurements of extracellular protein revealed complex changes in amount, whereby at 37 and 45°C, two maxima occurred at approximately $\mu = 0.1$ and 0.2 h^{-1}, but overall the amount fell with increased growth rates. At 50°C, extracellular protein rose to a single maximum at $\mu = 0.2$ and thereafter fell to low levels. The highest amount of extracellular protein detected in continuous cultures occurred at 45°C reaching values of 400 mg L^{-1} compared with 95 and 100 mg L^{-1} at 37 and 50°C, respectively. Maximum growth rates allowed by each temperature reflected the truly thermophilic nature of *S. thermoviolaceus*, such that at 50°C a growth rate of 0.5 h^{-1} could be achieved, whereas at 37°C, a maximum of only 0.3 h^{-1} was recorded (*52*). However, even at the lower temperatures, these growth rates compare favorably with work reported for erythromycin production by the mesophile *Streptomyces erythraeus*. This streptomycete also produced antibiotic in phosphate-limited continuous culture, and amounts synthesized increased with a growth rate up to $\mu = 0.1$ h^{-1}. However, μmax was only 0.17 h^{-1} (*53*). A number of interesting conclusions stem from the work described above for *S. thermoviolaceus*. Antibiotic synthesis occurs at relatively high temperatures, and therefore, *S. thermoviolaceus* must have a polyketide pathway that is thermostable. Growth is rapid at high temperatures, which means that *S. thermoviolaceus* reaches the phase of secondary metabolism much faster than mesophilic species. Figure 1 illustrates this by comparing growth and granaticin production in *S. thermoviolaceus* at 50°C with synthesis of the related antibiotic actinorhodin in *S. coelicolor* at 30°C. Growth and antibiotic synthesis in the thermophile are complete after approx 12 h, whereas the mesophile takes approx 70 h to reach the same stage. This could have some economic benefit in faster production rates, particularly in view of the rapid developments in cloning and sequencing of antibiotic biosynthetic pathways in streptomycetes, and more rapid turn around times of industrial-scale fermentations. Further economies may be possible because of the lower energy requirement for cooling of large-scale fermentations.

The reason for the secretion of extracellular proteins that are associated with a change in growth rate is unclear, but could be owing to cell lysis

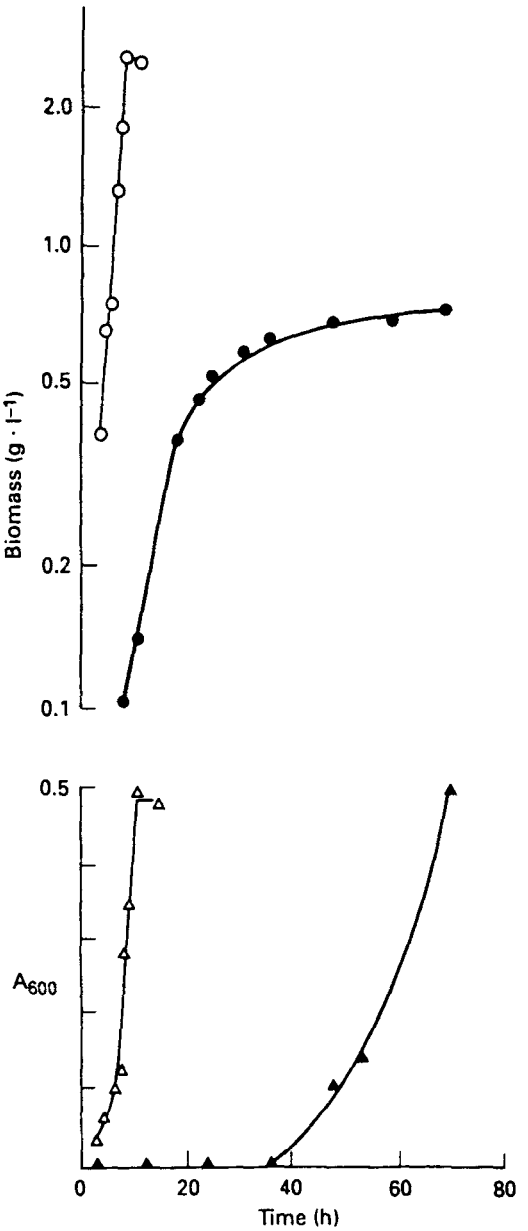

Fig. 1. Comparison between antibiotic production by a mesophilic streptomycete with that by a thermophilic species. Actinorhodin production (▲) was measured spectrophotometrically at 600 nm in cell-free supernatants prepared at different stages of fermentation during growth (●) of *S. coelicolor* at 30°C. Granaticin, (△) a closely related antibiotic, was monitored in the same way for *S. thermoviolaceus* grown (○) at 50°C. Taken from ref. (*31*) with permission.

associated with mechanisms of antibiotic efflux or to some trigger that results in active protein secretion. Lysis can be discounted because protein secretion occurs under conditions that are optimal for growth and at time when there is no apparent nutritional stress on the cells. Furthermore, assays for cytosolic enzymes failed to reveal extracellular activity arguing for secretion of specific proteins rather than lysis of the mycelium. A role for antibiotic efflux causing leakage of cell proteins can also be discounted because similar biphasic growth kinetics and patterns of protein secretion occur in the nongranaticin-producing variant *S. thermoviolaceus* subsp. *apingens*. Recent work indicates that high-growth temperature has an indirect role in the causation of biphasic growth. The respiratory chain of *S. thermoviolaceus* is not particularly thermotolerant (*54*) particularly at the level of NADH oxidation; in addition, two terminal oxidases can be present. One is an *a*-type oxidase, and the other is cytochrome oxidase *d*, which is characterized by its resistance to KCN. Respiratory chain activity of *S. thermoviolaceus* was probed during the first rapid and second slower growth phases by measuring NADH oxidation in membrane preparations exposed to a range of cyanide concentrations. Modulation of terminal oxidase activity was apparent, so that membranes prepared from cultures in the antibiotic-producing phase were less sensitive to KCN than those prepared from the early exponential phase (*55*). The probable reason for this difference was the synthesis of cytochrome oxidase *d* during later stages of growth. Cytochrome *d* is often induced under conditions of oxygen limitation (*56*). Therefore, a contributory factor to induction of the alternative oxidase may be oxygen limitation, which arises at a critical mycelial mass whereby respiratory activity exceeds supply. This could arise because of the lower oxygen solubility that occurs with increased temperature or the formation of mycelial aggregates that are large enough to form oxygen gradients, which ensure adequate aeration to some hyphae but not to others. It is possible that a combination of the two may also occur. The net effect to the growing mycelium will be energy limitation, which may be responsible for reduced growth rate and protein secretion. Some support for this comes from the observation that the inflexion of biphasic growth at 45°C occurs at a similar biomass concentration irrespective of the carbon source being utilized (*55*). These data are of relevance to those actinomycetes, particularly streptomycetes, involved in natural product (e.g., antibiotics) formation. Oxygen limitation may be a factor in diverting substrates, normally destined for oxidation and energy production, into secondary metabolite producing pathways.

SUMMARY

Actinomycetes are widespread in natural environments, and mesophilic species have been and continue to be extensively screened for their potential for producing useful natural products. Because of this, it is be-

coming more difficult to recover truly novel isolates. However, there is considerable scope for extending screening programs to unusual habitats where strains possessing novel properties can be isolated. Work described here on thermophilic actinomycetes illustrates the need to devise appropriate isolation protocols that when applied to environmental samples may reveal thus far unrealized diversity. The isolation of *S. thermoautotrophicus* exemplifies the potential for identifying strains that are not only novel actinomycetes, but also display metabolic properties that differ from established groups of bacteria. There is also considerable scope here for identifying novel natural products synthesized in response to unusual environmental conditions, such as elevated temperature, acid soils, salt marshes, or alkaline conditions.

Because of their habitats, there is also considerable potential for screening thermophilic actinomycetes for thermostable polymer-degrading enzymes. What little is known in this area would suggest that there may be possible synergistic applications of enzyme consortia for biodegradation of complex chemicals at high temperatures. It should also be noted that thermophilic actinomycetes, alongside other microorganisms, are already involved in a biotechnological application that involves the production of mushroom compost. This low-technology process may point the way forward for applications of thermophilic actinomycetes to solid substrate fermentations for biotransformations, production of novel hydrolases, or biodegradation of toxic compounds. Studies of growth physiology in *S. thermoviolaceus* herald a potential at least for the more rapid production of secondary metabolites. This organism also has a thermostable polyketide pathway that, through the applications of molecular genetic techniques, could possibly be used as a base for the synthesis of commercially important polyketide-derived antibiotics at high growth temperatures. The optimum temperature of 45°C for growth and antibiotic synthesis in this organism is also optimal for minimum input of energy for cooling large-scale commercial fermentations. Finally, studies of bacteria from environmental extremes, like thermophilic actinomycetes, can also challenge current understanding and concepts gained from traditionally studied organisms.

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