

REVIEW

Ecological Approach to Unravel Streptomyces Diversity as an Unsurpassed Sources of Natural Bioactive Products

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Search and discovery for natural bioactive products have been so important to control the emergence of antibiotic resistant microbial pathogens. Therefore, novel microorganisms that produce such metabolites is extremely needed. The capacity of members of the genus *Streptomyces* to produce commercially significant bioactive metabolites, notably antibiotics remains unsurpassed. However, it is acknowledged that discovering commercially useful secondary metabolites from streptomycetes is becoming more difficult due to lack of knowledge on the ecology and complexity of streptomycete systematics. In fact, those are fundamental aspects for developing strategy and method for isolation. In order to devise an appropriate program for successful selective isolation of streptomycetes, it is fundamentally important to understand their occurrence and activity in nature. A multistep extraction procedure designed for representative sampling, called dispersion, and differential centrifugation technique in combination with the incorporation of antibiotics into isolation media has become one of the most important selective method for the isolation of streptomycetes from natural habitats. The availability of new procedures to selectively isolate representative of streptomycetes from natural habitats opens up the possibility to determine the extent of streptomycete diversity from various habitats. Hence, the capacity of well characterized streptomycete isolates to produce commercial novel active metabolites could be further assessed appropriately.

Key words: ecological approach, streptomycete, diversity, natural bioactive

The search and discovery of new microorganisms that produce novel secondary metabolites is extremely important not least because of the need to find new pharmacologically active compounds to control the emergence of antibiotic resistant microbial pathogens (Bérdy 1995; Demain 1998; Demain and Elander 1999). It is widely acknowledged that some microorganisms are better sources of bioactive compounds than others. Amongst bacteria, the ability of members of the genus *Streptomyces* to produce commercially significant, pharmacologically active metabolites, notably antibiotics, remains unsurpassed (Bérdy 1995; Sanglier *et al.* 1996; Garrity and Holt 2001). However, it is becoming increasingly difficult to discover commercially useful secondary metabolites from these organisms as known streptomycetes are being isolated and screened with increasing frequency with the result that the same kinds of bioactive compounds are being rediscovered at great expense. This situation raises the question whether streptomycetes are exhausted as a source of new bioactive compounds and hence should lose their pre-eminence in screening programmes designed to detect novel natural products. To some extent, the answer to this question depends on the extent of the untapped taxonomic and genetic diversity that is encompassed in the genus at specific and infraspecific levels.

The primary aim of this review is to unveil the potential of the members of the genus *Streptomyces* as an unsurpassed source of bioactive products as well as the constraint that usually prevent the successful discovery of novel strain that

produce commercial bioactive product. The strategy and method developed on the basis of ecological and taxonomical selective isolation approach in order to overcome the problems of discovering novel bioactive producing streptomycetes are therefore also discussed accordingly.

Study of Streptomyces Diversity in Natural Habitats

The analysis of DNA extracted from environmental habitats shows that the genetic diversity of microorganisms is much greater in natural habitats than was previously recognized (Embley and Stackebrandt 1997; Head *et al.* 1998; Bull *et al.* 2000). The genus *Streptomyces* accommodates an unusually high degree of natural diversity with almost 600 validly described species (Goodfellow *et al.* 2007). Nevertheless, a steady flow of new streptomycete species are being described to accommodate either organisms isolated from diverse habitats (Kim *et al.* 1998; Al-Tai *et al.* 1999; Kim *et al.* 1999, 2000; Sembiring *et al.* 2000; Goodfellow *et al.* 2007; Ambarwati *et al.* 2009) or existing strains redescribed in the light of the application of modern taxonomic techniques (Labeda and Lyons 1991a,b; Labeda *et al.* 1997). It is clear from such studies that new streptomycete species should be circumscribed using a combination of genotypic and phenotypic data and that strains isolated from unexplored habitats are likely to form new centres of taxonomic variation. There is a strong circumstantial evidence that the discovery of previously unknown natural products occurs when novel organisms are examined in either established or new pharmacological

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screening programmes (Nolan and Cross 1988; Omura 1992; Woodruff 1999).

It is highly probable that the genus *Streptomyces* is underspeciated partly because of the historical difficulties in isolating and characterising a representative sample of the streptomycete community found in natural habitats. However, the availability of new procedures to selectively isolate and characterise representative of streptomycetes from natural habitats opens up the possibility of determining the extent of streptomycete diversity associated with neglected habitats, such as the rhizosphere of tropical trees (Sembiring *et al.* 2000; Ambarwati *et al.* 2009). It is becoming increasingly apparent that streptomycetes are widely distributed in the root systems of a broad range of plants (Upton 1994; Katsifas *et al.* 1999; Atalan *et al.* 2000; Sembiring *et al.* 2000) though little is known about the extent of their taxonomic diversity, activities or interactions with other organisms found in and around plant roots. Nevertheless, there is evidence that streptomycetes or their products can be used to suppress root-infecting fungi *in vivo* (Lui *et al.* 1995; You *et al.* 1996; Trejo-Estrada *et al.* 1998a).

An example of biosystematic studies on members of three putatively novel *Streptomyces* species isolated from rhizosphere soil show that a coherent strategy is available to determine the species richness of cultivable streptomycetes isolated from environmental samples (Atalan *et al.* 2000). Representative strains from selective isolation plates can be grown on oatmeal and peptone-yeast extract-iron agars and assigned to groups based on aerial spore mass colour, substrate mycelial pigmentation, the colour of any diffusible pigments and the ability to produce melanin pigments. The resultant colour-groups can be evaluated by examining representative strains by using Curie-point pyrolysis mass spectrometry (Goodfellow *et al.* 1997a; Sembiring *et al.* 2000) and/or by 16S rDNA sequencing and DNA:DNA relatedness studies (Goodfellow *et al.* 1997b). A similar strategy has been used to highlight potentially novel rhodococci that were selectively isolated from deep sea sediments in the North-West Pacific Ocean (Colquhoun *et al.* 1998a,b, 2000).

Ecology and Strategy to Selectively Isolate Streptomycetes

Little is known about the geographical distribution of *Streptomyces* species (Goodfellow and Simpson 1987; Goodfellow and O'Donnell 1989; Bull *et al.* 1992) or about fluxes in streptomycete populations due to seasonal and climatic changes or to human intervention as in agriculture and farming practices (Atalan 1993; Upton 1994). This lack of knowledge can be partially attributed to the complexity of streptomycete systematics, notably to the lack of reliable identification schemes. The identification of streptomycetes below the genus level remains difficult and has been rarely attempted in ecological studies (Goodfellow and Dickinson 1985; Upton 1994; Manfio 1995; Atalan *et al.* 2000) even with the availability of computer-assisted identification procedures (Williams *et al.* 1983; Langham *et al.* 1989; Kämpfer and Kroppenstedt 1991).

The literature on the occurrence and activity of streptomycetes in nature is as extensive as it is diffuse (Williams 1982; Goodfellow and Williams 1983; Williams *et al.* 1984a,b; Goodfellow and Simpson 1987; McCarthy and Williams 1990; Korn-Wendisch and Kutner 1992). However, streptomycetes are common in both aquatic and terrestrial environments; most are strict saprophytes though members of a few species form parasitic associations with animals and plants. Little is known about the role of streptomycetes in natural habitats though composts, fodder and soil seem to be primary reservoirs.

Innumerable "non-selective" media have been recommended for the isolation of streptomycetes (Williams and Davies 1965; Williams *et al.* 1984a). Many of these contain glucose, glycerol, mannitol, or starch as the carbon source and arginine or asparagine as the nitrogen source. Chitin has also frequently been used as a source of carbon and nitrogen. Such "non-selective" media are now known to favour the isolation of a narrow ranges of streptomycetes and do not support the growth of actinomycetes with more exacting growth requirements (Cross *et al.* 1976; Williams *et al.* 1984a). Selective isolation procedures are necessary to determine the numbers and types of streptomycetes occurring in natural habitats.

Selective media favour the growth of target microorganisms but not that of unwanted organisms. A number of approaches based on some aspect of the biology of individuals or groups of organisms can be used to selectively isolate actinomycetes from environmental samples. The organisms may be selected by plating serial dilutions of environmental samples onto nutrient media containing compounds which inhibit the growth of unwanted bacteria but not that of the target streptomycetes, by enriching the environmental substrate prior to selective isolation or by treating it using either chemical and/or physical methods which favour the isolation of streptomycetes but not that of unwanted bacteria and fungi.

The incorporation of antibiotics into isolation media has become one of the most important selective techniques for the isolation of streptomycetes (Porter *et al.* 1960; Gregory and Lacey 1963; Williams and Davies 1965; Williams and Mayfield 1971; Orchard and Goodfellow 1974; Labeda and Shearer 1990). The antifungal antibiotics cycloheximide and nystatin are routinely incorporated into media selective for streptomycetes, at approximately 50 µg ml⁻¹ each, to eliminate or control the growth of fungi on isolation plates. Media supplemented with antibacterial antibiotics are often used to good effect though streptomycete counts as well as those of unwanted bacteria may be reduced (Williams and Davies 1965; Davies and Williams 1970).

It is always difficult to know which antibiotic or combination of antibiotics are likely to be the most effective for the isolation of target organisms. One approach which has been applied with some success is to determine the antibiotic sensitivity patterns of representatives of a specific taxon and to supplement media with antibiotic(s) that inhibit unwanted bacteria but not that of the target streptomycetes. Williams and Davies (1965) screened members of 45 *Streptomyces* spp. against four antibiotics at five different

concentrations and found that the least inhibitory antibiotics were polymixin B sulphate ($5.0 \mu\text{g ml}^{-1}$) and sodium penicillin ($1.0 \mu\text{g ml}^{-1}$). They supplemented starch-casein agar with these antibiotics and found a decrease in the total number of streptomycetes from soil though the plates were cleaner and streptomycete colonies easier to recognise and isolate than on control plates lacking antibacterial antibiotics.

The high streptomycetes counts associated with habitats such as soil need to be interpreted with care as most colonies growing on isolation plates originate from spores. The growth of streptomycetes in soil is similar to that of many other microorganisms in this habitat where supplies of nutrients are discontinuous. It seems that streptomycetes live in soil for long periods as arthrospores that germinate in the presence of exogenous nutrients, the lack of which prevents germination of most or all spores added to sterile soil (Mayfield *et al.* 1972). These investigators estimated the mean doubling time of streptomycetes in soil to be 1.7 days. This protracted doubling time probably reflects the stop-go nature of the streptomycete life-cycle. The specific growth rates and generation times of streptomycetes grown in batch culture are roughly intermediate between those of bacteria and fungi (Flowers and Williams 1977).

The survival capacity of streptomycete spores appear to be greater than that of hyphae (Williams *et al.* 1972). The walls of spores are usually thicker than those of hyphae (Sharples and Williams 1976) and are also more hydrophobic (Ruddick and Williams 1972) due to the presence of an outer sheath that envelopes the spore wall (Williams *et al.* 1973). Streptomycete spores have a net negative surface charge at low pH levels (Douglas *et al.* 1971), a relatively low endogenous metabolism (Ensign 1978) and generally show more resistance to heat than corresponding hyphae (Goodfellow and Simpson 1987). They are dispersed above soil by wind or rain (Lloyd 1969) and within soil by arthropods and water movements (Ruddick and Williams 1972).

The major factors governing the distribution and activity of streptomycetes in soil are nutrient availability, moisture content, temperature and pH though soil type and seasonal change also have an influence (Williams *et al.* 1972; Williams 1978; Atalan 1993; Upton 1994). Streptomycetes can grow in soil at low oxygen levels, but not when carbon dioxide concentrations exceed 10%. In arid soils, streptomycete counts decrease sharply at moisture tensions above pF 4.0, but their relative proportion to other bacteria may be greater as their spores are more resistant to desiccation than vegetative cells of bacteria. Optimal counts from neutral soils and optimal radial growth of streptomycetes inoculated into sterile soil occur at moisture tensions between pF 1.5 and 2.5. At these tensions, soil pores are partially filled with available water but still contain sufficient air for the growth of the aerobic microbiota. Halophilic (Hunter *et al.* 1981) and osmophilic streptomycetes (Wong and Griffen 1974) have been reported.

Soil reaction is an important factor determining the distribution and activity of streptomycetes. Acidophilic and neutrotolerant streptomycetes, which grow between pH 3.5 and 7.5 but optimally around pH 5.5, are common in acidic soils (Williams *et al.* 1971; Khan and Williams 1975;

Goodfellow and Dawson 1978; Goodfellow and Simpson 1987). These organisms produce chitinases (Williams and Robinson 1981) and diastases (Williams and Flower 1978) with pH optima lower than those of en-zymes from neutrophilic streptomycetes which grow between pH 5.5 and 8.0, but optimally around 7.2. The presence of low numbers of neutrophilic streptomycetes in acidic soils has been attributed to their ability to grow in less acidic microsites (Williams and Mayfield 1971). It has been shown that when nitrogen containing substrates, such as chitin or dead fungal mycelium, are added to poorly buffered acidic soil, a succession of acidophilic to neutrophilic streptomycetes occurs that parallels ammonification and the resultant rise in pH (Williams and Robinson 1981).

Little is known about the growth of most streptomycetes *in situ*. It seems unlikely that they grow optimally in temperate soils as most strains are mesophilic under laboratory conditions. However, temperature can indirectly be implicated in examples of the influence of seasonal and climatic factors in the size and composition of streptomycete populations. It has been reported that streptomycete counts in grassland were highest in summer and that the distribution of "*Streptomyces malachiticus*" is restricted to subtropical and tropical soils (Küster 1976).

Clay and humic colloids can influence the activity of streptomycetes at the micro-environmental level. Streptomycete spores are readily adsorbed to kaolin but not to montmorillonite except at low pH (Ruddick and Williams 1972). Addition of calcium montmorillonite or calcium humate to cultures of streptomycetes can accelerate their growth and respiration (Mara and Oragui 1981). It has also been shown that sites of adsorption with humic material can lead to microsites of increased pH in acidic soils (Williams and Mayfield 1971).

Streptomycete as a Potential Source of Natural Bioactive Products

Wide range of marketed microbial agents with therapeutic which are produced by the streptocetes including antibacteria (cephamycins, carbapenems, clindamycin, quinupristin, and streptomycin), antifungal (nystatin, cycloheximide), antineoplastics (daunorubicin, doxorubicin), immunomodulator (tacrolimus, rapamycin), and antiparasitic (ivermectin, abamectin, doramectin, moxidectin (Kuo and Garrity 2002). However, in general, streptomycetes are not considered to have a significant role in plant root systems (Williams *et al.* 1984b). However, it is now apparent that streptomycetes are widely distributed in the root systems of diverse plants (Rangaswami and Vasantharajan 1962; Bernhard 1967; Watson and Williams 1974; Vrugink 1976; Buti 1978; Miller *et al.* 1990; Sardi *et al.* 1992; Upton 1994; Katsifas *et al.* 1999; Atalan *et al.* 2000) though little is known about the extent of their diversity, activities, or interactions with other organisms in the root environment. Positive rhizosphere effects have been reported for streptomycetes in several root systems, such as those of maize, perennial ryegrass, soya, tomato, and wheat (Abraham and Herr 1964; Upton 1994).

There is a growing interest in using members of the streptomycete rhizosphere community to enhance plant growth and production, and to inhibit root infecting fungi (Hettiarachi and Penninckx 1990; Trejo-Estrada *et al.* 1998a,b). Two mechanisms have been proposed to explain the inhibition of fungal pathogens in the rhizosphere by biocontrol agents. Antibiosis occurs when one or more diffusible compounds inhibit growth or development changes in the pathogen thereby impairing its ability to colonise the rhizosphere and establish disease. Mycoparasitism is a different process which is initiated by physical destruction of the fungal cell wall mediated by the action of hydrolytic enzymes produced by the biocontrol agent (Adams 1990).

Most actinomycetes considered to suppress the growth of root infecting fungi are streptomycetes (Table 1).

Antibiotics produced by actinomycetes have been used directly or assumed to be responsible for the biocontrol potential of the producing strains. Examples of such metabolites include aminoglycosides (Qin *et al.* 1994), macrolide benzoquinones (Rothrock and Gottlieb 1984), nucleosides (Hwang *et al.* 1994) and polyenes (Smith *et al.* 1990; Raatikainen *et al.* 1994). *Streptomyces violaceusniger* strain YCED-9 is an antifungal biocontrol agent which produces three different antibiotics, namely geldanamycin, nigericin, and a complex of macrocyclic lactone antibiotics (Trejo-Estrada *et al.* 1998a,b). This organism, which was isolated from soil by Crawford *et al.* (1993), was selected for its potential to suppress damping-off disease of lettuce caused by *Pythium ultimum*, and for its ability to antagonize the growth of many fungal pathogens *in vitro* and *in vivo* (Crawford *et al.* 1993; Crawford 1996). *Streptomyces* strain

Table 1 Actinomycetes reported to be antagonistic towards fungal root pathogens

Actinomycete genus/species	Fungal pathogen	Reference
<i>Actinoplanes missouriensis</i>	<i>Aphanomyces</i> sp. <i>Phytophthora</i> sp. <i>Pythium</i> sp.	Sutherland <i>et al.</i> (1984)
<i>Actinoplanes</i>	<i>Phytophthora</i> sp. <i>Phytophthora</i> sp. <i>Phytophthora capsici</i> <i>Pythium</i> spp.	Sneh <i>et al.</i> (1977) Sutherland and Lockwood (1984) Sutherland and Papavizas (1991) Khan <i>et al.</i> (1993)
<i>Micromonospora</i>	<i>Phytophthora</i> sp.	Sutherland and Lockwood (1984)
<i>Rhodococcus</i> <i>S. diastatochromogenes</i> <i>S. griseoalbus</i>	<i>Gaeumannomyces graminis</i> <i>Pythium debaryanum</i> <i>Phellinus weirii</i> <i>Fomes annosus</i> <i>Phytophthora cinnamomi</i>	Renwick <i>et al.</i> (1991) Kaspari (1973) Rose <i>et al.</i> (1980)
<i>S. griseus</i>	<i>Rhizoctonia solani</i> <i>Phomopsis sclerotoides</i>	Merriman <i>et al.</i> (1974a, 1974b) Ebben and Spencer (1978)
<i>S. hygrosopicus</i> subsp. <i>geldanus</i> <i>S. violaceusniger</i> strain A50	<i>Rhizoctonia</i> sp. <i>Botryosphaeria dothidea</i> <i>Phytophthora capsici</i> <i>Rhizoctonia solani</i>	Rothrock and Gottlieb (1984) Hwang <i>et al.</i> (1994)
<i>S. violaceusniger</i> YCED-9 <i>Streptomyces</i>	<i>Rhizoctonia solani</i> <i>Fusarium oxysporum</i> <i>Phymatotrichum omnivorum</i> <i>Rhizoctonia solani</i> <i>Verticillium alboatrum</i> <i>Gaeumannomyces graminis</i> <i>Rhizoctonia bataticola</i> <i>Aspergillus</i> spp. 'Wood infecting fungi' <i>Phytophthora cinnamomi</i> <i>Fusarium oxysporum</i> <i>Fusarium oxysporum</i> <i>Fusarium moniliforme</i> <i>Sclerotium rolfsii</i> <i>Fusarium oxysporum</i> <i>Aspergillus parasiticus</i> <i>Fusarium tricinctum</i>	Trejo-Estrada <i>et al.</i> (1998a) Whaley and Boyle (1967) Smiley (1978a, 1978b) Sing and Mehrota (1980) Stabi <i>et al.</i> (1980) Blanchette <i>et al.</i> (1981) Murray (1987) Sabaou and Bounagu (1987) Huber <i>et al.</i> (1989) Kalappanavar and Hiremath (1990) Plakshappa <i>et al.</i> (1990) Chung and Hong (1991) Borghetti <i>et al.</i> (1992)
<i>Streptomyces</i> strain 385	<i>Fusarium oxysporum</i>	Singh <i>et al.</i> (1999)
Not stated	<i>Phytophthora</i> spp. <i>Rhizoctonia solani</i> <i>Fusarium culmorum</i> <i>Fusarium udum</i> <i>Corticium salmonicolor</i> <i>Phytophthora capsici</i> <i>Phytophthora cinnamomi</i> <i>Fusarium oxysporum</i> <i>Pythium ultimum</i>	Keast and Tonkin (1983) Kundu and Nandi (1985) Kempf and Wolf (1989) Guar and Sharma (1991) Joseph <i>et al.</i> (1991) Ahn and Hwang (1992) Stirling <i>et al.</i> (1992) Abdel-Moneim <i>et al.</i> (1993) Crawford <i>et al.</i> (1993)

385 suppresses fusarium wilt of cucumber (*Cucumis sativus*) caused by *Fusarium oxysporum* when used in combination with *Paenibacillus* strain 300 (Singh *et al.* 1999).

Natural bioactive substances, notably which are produced by microorganisms have been the subject for many studies due to their importance in the fields of both medicine and agriculture. Such natural microbial bioactive products are including antibiotics, anticancer, antiviral, immunomodulator as well as antiparasitic. In the field of medicine, antibiotics have been used as the main agents to control the emergence of antibiotic resistant microbial pathogens, while in the field of agriculture antibiotics and antihelminth have also been utilized to control plant pathogen microbes as well as plant pest nematodes, respectively. Since amongst bacteria, the ability of members of the genus *Streptomyces* to produce commercially significant, pharmacologically active metabolites, notably antibiotics, remains unsurpassed, it is reasonable that streptomycete diversity has been among the most important target for search and discovery for this bioactive natural products. Therefore, the comprehensive study of the streptomycete biodiversity in natural habitats could provide a very useful information to design screening program in order to obtain the best producer strains among the member of the genus *Streptomyces*.

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