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Enhancement of cyclosporin production in a *Tolypocladium inflatum* strain after epichlorohydrin treatment

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Summary

Following treatment of conidia of the cyclosporin producer fungus, *Tolypocladium inflatum*, with 0.15 M epichlorohydrin, strain M6 was isolated. The new strain exhibited a similar growth rate to the parent organism but more extensive conidiation and several-fold higher overall cyclosporin production. Strain M6 reached titres of 318 mg l^{-1} cyclosporin A in agar cultures, whereas in liquid medium it produced 140 mg l^{-1} cyclosporin A and 68 mg l^{-1} cyclosporin C. It also maintained a steady volumetric productivity of 0.48 mg l^{-1} h^{-1} cyclosporin A over 2 weeks of submerged cultivation in maltose-based semisynthetic medium. The new strain holds potential for improved cyclosporin production due to the superior titres and demonstrated capacity to sustain elevated production of cyclosporin for periods greater than the wild type.

Cyclosporin A; Cyclosporin C; *Tolypocladium inflatum*; Epichlorohydrin; Genetic variant

Introduction

The immunosuppressive agent cyclosporin A (also spelled cyclosporine, ciclosporine; commercial name: Sandimmun^{TM}), discovered and developed at

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Sandoz SA (Basel, Switzerland) in the early 1970s is the most prominent among several eleven-membered cyclic peptides (cyclosporins) produced in submerged cultivation by the hyphomycete *Tolypocladium inflatum* Gams (Kobel and Traber, 1982). Cyclosporin A, initially described as a weak antifungal antibiotic (Dreyfuss et al., 1976) is currently enjoying increased clinical use, particularly in the area of transplant surgery (Borel, 1984), due to its highly specific immunosuppressive action (Wiesinger and Borel, 1979). Despite the prominence of the drug in the clinical literature, there are few reports on the biology and process engineering of the fungal production of cyclosporin A (Dreyfuss et al., 1976; Kobel and Traber, 1982; Agathos et al., 1986; 1987; Chun and Agathos, 1989; Lee and Agathos, 1989). No genetic approaches towards increasing the biotechnological value of *T. inflatum* have been reported.

A recent study (Agathos et al., 1986) examined the carbon and nitrogen nutrition of the producer fungus in relation to its secondary metabolism, i.e., the formation of cyclosporins. In addition, we have initiated genetic studies of *Tolypocladium* sp. to determine their antibiotic tolerance and their protoplasting and regeneration efficiency (Agathos et al., 1986). We are now reporting on the development and preliminary characterization of a *T. inflatum* strain capable of markedly increased and sustained cyclosporin A production.

Materials and Methods

Organisms and growth conditions

The parent strain of *Tolypocladium inflatum* (ATCC 34921) was obtained from the American Type Culture Collection, Rockville, Maryland. Parent and M6 were maintained on agar slants of MYA medium (Kobel et al., 1983) at 4° C. The medium contained, per liter of distilled water: malt extract, 20 g; yeast extract, 4 g; agar, 20 g (pH 5.7).

Seed inoculum for shake flask cultivations was prepared as previously described (Agathos et al., 1986), using suspensions from MY agar slants to inoculate the seed liquid medium MY (same as MYA, without agar). Seed cultivations were carried out in 250 ml Erlenmeyer flasks containing 50 ml of MY medium shaken reciprocally at 200 rpm and 27° C for 72 h.

Shake flask cultivations for cyclosporin production were conducted in the semi-synthetic medium (SSM) as reported previously (Agathos et al., 1986), with 10 ml inoculum in 100 ml medium, using 500 ml Erlenmeyer flasks. The SSM, per liter of distilled water, consisted of: maltose or sorbose, 30 or 50 g; Bacto-peptone, 10 g; KCl, 2.5 g; KH_2PO_4, 5 g (pH 5.7). All media components except complex ingredients were of analytical grade. Complex media ingredients and agar were from Difco Laboratories, Detroit, Michigan.

Determinations of biomass, carbon source and cyclosporins

Mycelial biomass was estimated as dry cell weight. Carbon source utilization was followed with an anthrone method (Weiner, 1978). Cyclosporins (A and C) were

determined by the methodology reported by us previously (Agathos et al., 1986). Briefly, the reaction mixture was extracted with equal volumes of butyl acetate over 18 h, the organic phase was separated by centrifugation, evaporated under N_2 gas and the residue subsequently redissolved in methanol. Cultures growing on agar medium were extracted similarly, after prior maceration of the solid mycelial mat, to which an approximately equal volume of butyl acetate was added. The fungal metabolites were estimated via an HPLC procedure (Carruthers et al., 1983) using as standards authentic cyclosporins, provided by Dr. D. Freeman (University of Western Ontario, Canada). All solvents used were of HPLC grade.

Epichlorohydrin treatment and strain (M6) isolation

Ten to twelve 5-mm agar "plugs" were removed from an agar culture of *T. inflatum* ATCC 34921 (MYA medium), placed into 50 ml of fresh liquid MY medium and shaken for about 72 h on a rotary shaker at 150 rpm and 27° C until ample sporulation was observed. In a variation of this sequence, 5 ml of a 24-h culture in liquid MY medium were transferred to 50 ml of Booth's sporulation methylcellulose medium (Booth, 1971) and incubated for 48 h on a rotary shaker at 150 rpm and 27° C, until extensive sporulation was seen. Alternatively, 5 ml of 6 to 8 day old liquid cultures grown in MY medium were transferred into 50 ml of fresh MY medium and the cultivation proceeded as above, with abundant production of conidia by the third day of incubation. The solution was subsequently filtered through simple laboratory tissue paper to separate the conidia from the mycelium. The conidia were washed with sterile distilled water, centrifuged at $5000 \times g$ for 10 min and washed again twice with sterile distilled water. After the second wash, the conidial pellet was suspended in 10 ml of 0.15 M epichlorohydrin and treated for 150 min. This dosage had been selected early in our mutagenesis program through a 50% survival curve based on concentration and period of treatment for each of three mutagens (epichlorohydrin, dimethyl sulfate and nitrosoguanidine) on each of four *Tolypocladium* strains (Agathos et al., 1986). The conidia were washed twice subsequently with sterile distilled water and plated, after appropriate dilutions, on minimal agar medium containing, per liter of distilled water: glucose, 0.5 g; sodium citrate, 0.5 g; K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; agar (purified), 15 g. In an attempt to increase the likelihood of obtaining amino acid auxotrophic mutants, the general method of Holliday (1956) was followed with minor modifications. After 18 h of incubation at 27° C, groups of four different amino acids were spotted on each minimal plate using 1 mg ml^{-1} amino acid solutions. A total of 14 amino acids were used. Colonies growing around any amino acid were isolated and examined for the corresponding amino acid requirement. Strain M6 was isolated from a 'ridge' of a colony growing around asparagine. Strain M6 as well as a number of other strains of *T. inflatum* isolated in this manner did not appear to be auxotrophic when plated back on minimal agar, i.e., they grew in the absence of the specific amino acid in the vicinity of which they had been initially isolated.

Results and Discussion

Comparison of T. inflatum ATCC 34921 and new strain M6 – morphology and growth rate

The major morphologic characteristics of colony appearance for the parent and the new strain of *T. inflatum* are summarized in Table 1. The most striking differences between M6 and wild type lay in the higher production of conidia and the abundance of exudate in the M6 strain. While this exudate did not contain any detectable levels of cyclosporins (as non-polar cyclic peptides, the cyclosporins are almost insoluble in aqueous media and, therefore, intracellular), the overall titre of cyclosporins from agar cultures of M6 was found to be several-fold higher than that of the wild type. After subculturing and using in submerged cultivations on semisynthetic medium, strain M6 produced almost four times greater volumetric titres of cyclosporin than the parental ATCC strain (Table 2), while maintaining profuse production of spores even under these cultivation conditions. The intense sporulation of M6, which is apparent even in the young peripheral zone of colonies on agar medium, may be related to its high cyclosporin productivity, as it is known that the process of differentiation is often associated with secondary metabolite production (Schaeffer, 1969).

There was no significant difference in the radial growth rate of M6 and its parental strain when grown in solid state (Table 1). Additionally, the growth rate

TABLE 1

COMPARISON OF *T. INFLATUM* STRAINS ATCC 34921 (WILD TYPE) AND M6 CULTURED ON MYA MEDIUM

Characteristic	Strain	
	Wild type	M6
Cultural morphology	Mycelia cottony; surface mildly undulating radially, with an umbonate central feature	Mycelia felt-like; marked surface undulations radially, outside a defined central area of irregular growth
Exudates	Little or none	Abundant discrete dispersed microdroplets, amber, appearing on day 6 and reaching maximum on day 14 of incubation
Microscopic features	Conidia and conidiophores numerous, located centrally around inoculum in the older areas; conidia and phialides typical of *T. inflatum*	Conidia and conidiophores very abundant, located uniformly across the colony, including the young peripheral zone; conidia and phialides typical of *T. inflatum*
Cyclosporin production	40 mg l^{-1}	318 mg l^{-1}
Relative radial growth rate	1	1

TABLE 2

CYCLOSPORIN A PRODUCTION AND MYCELIAL GROWTH OF *T. INFLATUM* STRAINS (WILD TYPE AND M6 STRAIN) IN MALTOSE-BASED LIQUID SSM MEDIUM CULTIVATION

	Strain	
	Wild type	M6
Specific growth rate, [a] h^{-1}	0.018	0.019
Volumetric production [b] of CyA, mg l^{-1}	41.2	140
Specific production [c] of CyA per biomass, mg g^{-1}	5.0	13.2
Volumetric productivity [a] of CyA, mg l^{-1} h^{-1}	0.17	0.48

[a] For initial 3-day exponential growth phase of the two strains.
[b] Maximal titre attained.
[c] Maximal titre per corresponding biomass.
[d] Averaged over productive period of bioprocess (day 5 to day 18).

was similar for the two isolates when cultivated in liquid semisynthetic medium (Table 2).

The striking difference in colony morphology between strain M6 and its parent is in line with numerous observations of wild type and mutant fungal forms: secondary metabolite hyperproducing mutants of filamentous fungi often appear quite different in gross morphology from the parental strain (Backus and Stauffer, 1955). Such visual contrasts are frequently used as a tool for the selection of overproducers among morphological mutants. On the other hand, the characteristic taxonomic features of *T. inflatum*, such as the swollen phialides and typical hyphal branching patterns (Gams, 1971) were evident in both parent and strain M6 (Table 1).

Although the original objective of our mutagenesis program was to obtain stable amino acid auxotrophs of *T. inflatum*, the methodology adopted (Holliday, 1956) was used only as a preliminary tool. It is recognized that the colonies growing adjacent to spotted amino acids would not necessarily be auxotrophic mutants for the respective amino acid of the spot. Nonetheless, even in the absence of more controlled methodologies that include replica plating (Roberts, 1959), the simple technique employed in our work might increase the likelihood of generating amino acid auxotrophs. Epichlorohydrin was employed as an alkylating mutagen known to induce amino acid auxotrophy in fungi (Esser and Kuenen, 1967; Fincham and Day, 1965). The strains isolated in our mutagenesis program, including M6, may have been unstable amino acid auxotrophs that reverted to prototrophy in the second generation. This possibility is likely because (a) all strains were isolated from the vicinity of amino acid spots whereas no cultures were evident outside the spotted areas of the agar plates after the mutagenesis treatment, and (b) amino acid-exacting nutritional mutants are often unstable among the hyphomycetes. Thus, M6 may represent an unstable asparagine auxotroph that had subsequently reverted to prototrophy. Similarly, mutant strains M5 and M1 generated in the same mutagenesis program were isolated after treatment with epichlorohydrin and dimethyl sulfate respectively and were isolated from the vicinity of a methionine and a serine spot respectively. These may also be presumed revertants from unstable

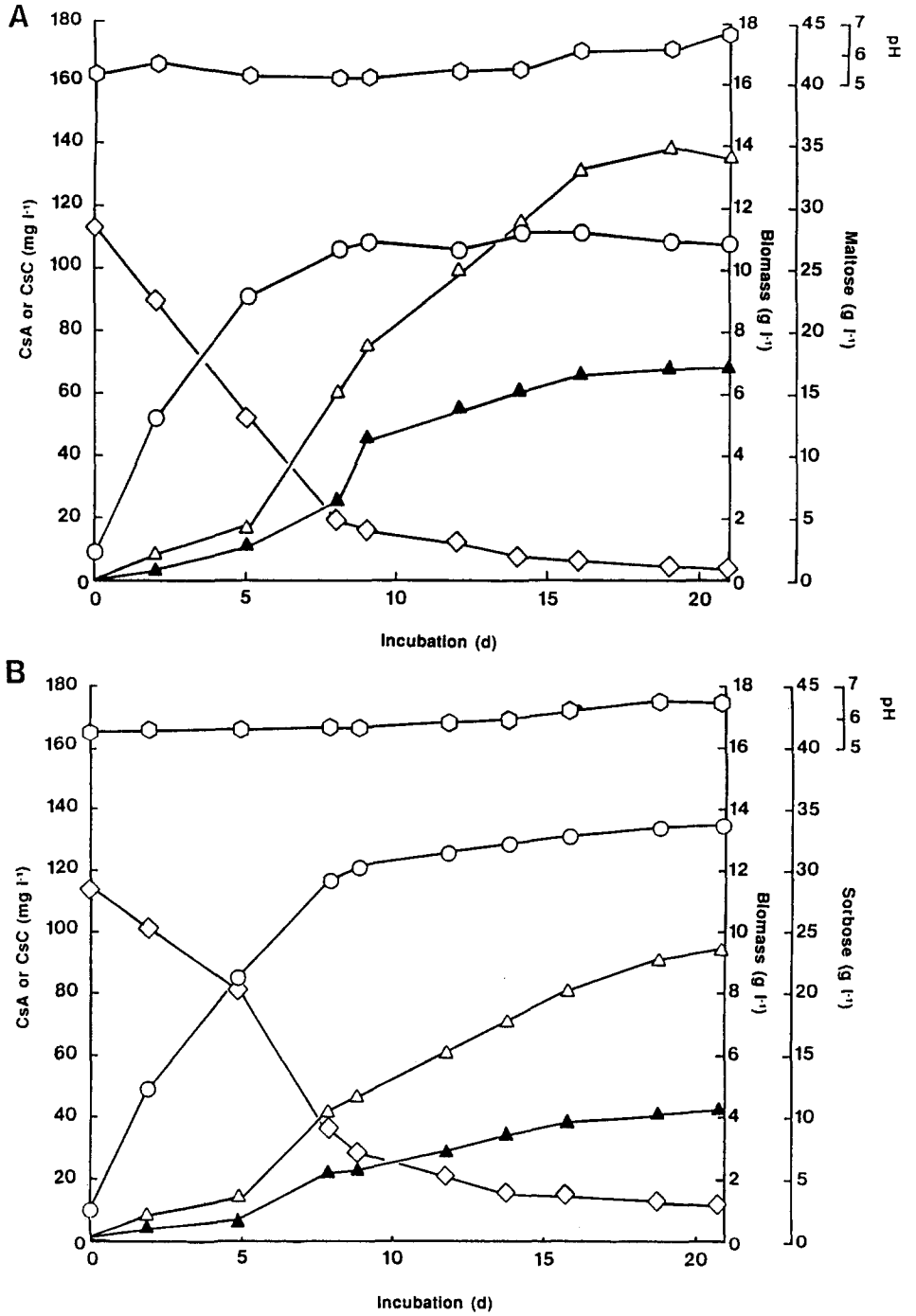

Fig. 1. Kinetic profile of a flask cultivation using *T. inflatum* strain M6 in SSM medium with 3% maltose (A) or 3% sorbose (B), as the carbon source. ○, biomass; ◇, carbon source; ⬡, pH; △, cyclosporin A, ▲ cyclosporin C.

amino acid auxotrophy back to prototrophy and represent, respectively, another cyclosporin hyperproducer (M5) and a cyclosporin non-producer (M1) (results not shown). Auxotroph revertant mutants often produce superior antibiotic titres and are, therefore, a significant class of mutants in strain improvement programs (Demain, 1973). Nevertheless, we cannot discount the possibility that the morphological (Table 1) and physiological (Table 2, Fig. 1) changes observed after epichlorohydrin treatment are the consequence of a pleiotropic effect on the organism and not due to reversion to prototrophy. The changes seen may have occurred in the parental population (a) spontaneously or (b) independent of any nutritional mutation. The possibility of a spontaneous mutation appears unlikely. We have attempted to plate out the parental organism without prior treatment and examine morphologically variable colonies, since frequently a parental fungal organism consists of strains that differ remarkably in the quantity of secondary metabolites produced. Our preliminary plating attempts have generated colonies with slightly different morphologies, but with no significant differences either in differentiation pattern (sporulation) or in cyclosporin production (variation $\pm 15\%$, i.e., within the limits of cyclosporin determination). The second possibility, i.e., that the changes observed in strain M6 have occurred as a result of a pleiotropic effect and not due to the sequence asparagine auxotrophy → prototrophy, cannot be excluded at this point.

Secondary metabolism

The feature that prompted our primary interest in strain M6 was its superior cyclosporin production. The time course of shake flask cultivations employing semisynthetic medium with maltose (3% w/v) or sorbose (3% w/v) as the carbon/energy source is shown in Fig. 1. While the growth of strain M6 was essentially complete (10.5 g dry cell weight (DCW) per litre) and 82% of maltose had been utilized by day 8 of the bioculturing, production of cyclosporins continued well beyond this point, reaching maximum titres (140 mg l^{-1} CyA and 68 mg l^{-1} CyC) on day 19 (Fig. 1A). By comparison, the parent strain produced only 41.2 mg l^{-1} CyA when grown in SSM medium containing 3% maltose (Agathos et al., 1986). Similar time course events can be seen in sorbose-based SSM medium although with this carbon source higher biomass levels (13.4 g l^{-1} DCW) and lower cyclosporin titres were reached (93 mg l^{-1} CyA and 40 mg l^{-1} CyC on day 21, Fig. 1B). The bioprocess kinetics of strain M6 are in contrast with the kinetic pattern displayed by *T. inflatum* ATCC 34921 (Agathos et al., 1986; 1987), in which both mycelial growth and cyclosporin formation were almost parallel and reached a plateau within 10 to 12 d. Also, the kinetic data reported by Dreyfuss et al. (1976), using an industrial strain of *T. inflatum*, show no substantial separation between trophophase and idiophase, unlike the behavior of strain M6. Hyphae fragmentation and lysis observed in cultivations of *T. inflatum* ATCC 34921 (Agathos et al., 1986; 1987) were almost absent throughout the length of the cultivations of *T. inflatum* M6 and indeed no reduction in mycelial biomass was recorded (Fig. 1A). This structural stability parallels and possibly accounts for the equally evident and considerably greater longevity of the cyclosporin biosynthetic apparatus in strain M6 compared

to the ATCC strain. Indeed, Fig. 1A reveals that an almost constant volumetric productivity averaging 0.48 mg l^{-1} h^{-1} CyA (calculated by taking slopes of the cyclosporin A production curve at successive time points) was sustained over a 13-d period starting from the point of growth deceleration (day 5) when there was a surge in secondary metabolite synthesis. A similar trend in long-term cyclosporin productivity was observed when sorbose was used (Fig. 1B), albeit this carbohydrate was a less favorable C-source for cyclosporin production in strain M6, in contrast with the parent strain (Agathos et al., 1986). In accordance with the principles provided by Demain (1982), a C-source supporting high biomass accumulation is often a poor C-source for secondary metabolism. On the other hand, the parental microbial process exhibited only one third of the volumetric productivity of strain M6 and this productivity was sustained for less than one week. Table 2 summarizes growth and production rate data calculated from batch cultivations of *T. inflatum* ATCC 34921 and *T. inflatum* M6.

The new strain of *T. inflatum* (M6) described in this communication appears to have considerable potential for use in an optimized process of cyclosporin production. This potential is justified not only by the several-fold higher cyclosporin titres obtained with M6 over the parent strain, but also by the steady long-term cyclosporin productivity of M6. While the significance of the longevity of antibiotic production has been addressed only recently in the literature (Gaucher et al., 1981; Agathos and Demain, 1983; 1988), a better understanding of this parameter could result in improved production processes. This latter feature of the new strain has suggested to us its use in conjunction with an appropriate feeding regime in order to attain even higher cyclosporin titres (Agathos et al., 1987). Because of the long-term stability of the cyclosporin biosynthetic apparatus, an additional pulse of maltose at day 8 of the batch bioculturing of M6 (when the original maltose had nearly been consumed) brought about a steady increase in secondary metabolite synthesis, attaining 537.5 mg l^{-1} cyclosporin A by day 16 of the cultivation (Agathos et al., 1987). Finally, this and other mutants of *T. inflatum* represent important biochemical and genetic tools for furthering our as yet limited understanding of the process of cyclosporin biosynthesis.

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