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Microbial Transformation of Aniline to Acetaminophen

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Abstract □ In order to obtain acetaminophen, a popular analgesic-antipyretic, through microbial p-hydroxylation and N-acetylation of aniline, various fungi and bacteria were screened. Among them, *Streptomyces* species were chosen for strain improvement by the use of interspecific protoplast fusion technique. Two interspecific fused strains were developed between *S. rimosus* (N-cetylation function) and *S. aureofaciens* (p-hydroxylation function) and also between *S. lividans* and *S. globisporus*. For efficient protoplast fusion and cell wall regeneration, various conditions were examined. In a typical experiment of mixed *S. rimosus* (pro^- his^-) and *S. aureofaciens* (ilv^-) protoplasts with 40% (w/v) polyethylene glycol 3350 (PEG) for 3 min gave 8.3×10^{-7} of fusion frequency. Treatment of mixed *S. lividans* ($pant^-$) and *S. globisporus* (leu^-) protoplasts with 50% (w/v) PEG for 3 min at 30℃ gave 1.2×10^{-6} of frequency. Among the fused strains, up to 40-50% increase in p-hydroxylation power was observed. To investigate the possibility of plasmid involvement in p-hydroxylation of acetanilide, plasmid curing was attempted. We found that cells treated with acriflavine (at the frequency of 100%) and cells regenerated from protoplsts of *S. aureofaciens* (2% frequency) lost their p-hydroxylation function.

Keywords □ Protoplast fusion, plasmid curing, p-hydroxylation of aniline

Microbial oxygenase systems, though often nonspecific to substrates, retain characteristics of stereoselectivity and regioselectivity, which make enyzme catalyzed processes attractive as alternatives to chemical routes of hydroxylations[1]. The prokaryotic genus *Streptomyces* is curious if not phenomenal in its ability to synthesize antibiotics of diverse chemical structures and modes of action. Genetic manipulation of these economically important microorganisms has commercial applications in yield improvement and in discovery of new intermediates and hybrid antibiotics with beneficial properties[2,3]. We believe techniques to generate hybrid antibiotics, such as mutasynthesis, protoplast fusion and recombinant DNA approach[4] are also applicable to microbial conversion of organic chemicals to biologically active pharmaceuticals. In order to obtain acetaminophen, a popular analgesic-antipyretic, from aniline through microbial p-hydroxylation and N-acetylation (Scheme 1), various fungi and bacteria were screened[5]. Among them, *Streptomyces* species were chosen for strain improvement. Thus we attempted to screen *Streptomyces* spp. for N-cetylation and p-hydroxylation activity of aniline to acetaminophen and to improve the screened strains by protoplast fusion. Furthermore we investigated the possibility of plasmid involvement in p-hydroxylation of aniline in *Streptomyces aureofaciens*.

EXPERIMENTAL METHODS

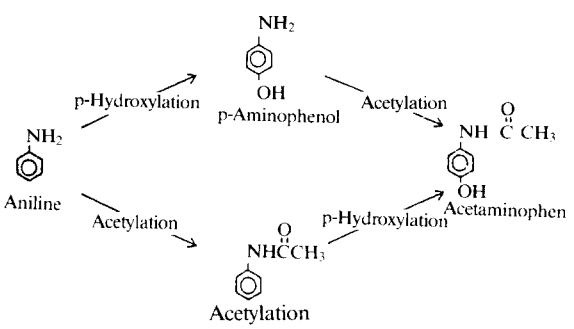
Bacterial strains and media

Various *Streptomyces* spp. used for p-hydroxylation of aniline are given in Table I. ATCC medium 5 was chosen for sporulation. For mutagenesis and selection of auxotrophs, complete medium (CM) and minimal medium (MM) were selected[6]. Theriault's screening medium (glucose 5%, soybean flour 0.5%, yeast extract 0.5%, KH_2PO_4 0.1%, K_2HPO_4 0.2%, NaCl 0.5%, pH 7.0)[7] was chosen for testing p-hydroxylation and N-acetylation of aniline. Medium S (glucose 1%, peptone 0.4%, yeast extract 0.4 %, $MgSO_4\cdot7H_2O$ 0.05%, KH_2PO_4 0.2%, K_2HPO_4 0.4 %) was used to cultivate the mycelium for protoplast formation. And for protoplast formation, protoplast stabilization and regeneration, P medium and R2

Table I. Microbial conversion of aniline and acetanilide

Species \ Substrates \ # Products \ Incubation periods		1 day		3 day		6 day		8 day	
		Acet	phen	Acet	phen	Acet	phen	Acet	phen*
Streptomyces aureofaciens	Aniline	+	−	+	+	+	+	+	+−
(ATCC 10762)	Acetanilide		+−		+		+		+
S. coelicolor	Aniline	+	−	+	−	+	−	+	−
(A3 (2). Hopwood)	Acetanilide		−		−		−		−
S. erythraeus	Aniline	+	−	+	−	+	−	+	−
(ATCC 11635)	Acetanilide		−		−		−		−
S. griseus	Aniline	+	−	+	+−	+	+−	+	+−
(NPRL B 2252)	Acetanilide		−		+−		+−		+−
S. lividans	Aniline	+	−	+	−	+	−	+	−
(IFO 12826)	Acetanilide		−		−		−		−
S. rimosus	Aniline	+	−	+	−	+	−	+	−
(ATCC 13224)	Acetanilide		−		−		−		−
S. venezuelae	Aniline	−	−	−	−	−	−	+	−
(ATCC 10712)	Acetanilide		−		−		−		−
S. violaceus-ruber	Aniline	+	−	+	−	+	−	+	−
(IFO 13385)	Acetanilide		−		−		−		−
S. globisporus	Aniline	−	−	−	+−	−	+	+	+
(ATCC 1829)	Acetanilide		−		+		+		+
S. albus	Aniline	+	−	+	−	+	−	+	−
(ATCC 21132)	Acetanilide		−		−		−		−

*: +,−; ability of product formation
Acet; Acetanilide, phen; Acetaminophen



Scheme 1 A possible biotransformation pathway of aniline to acetaminophen in microorganisms.

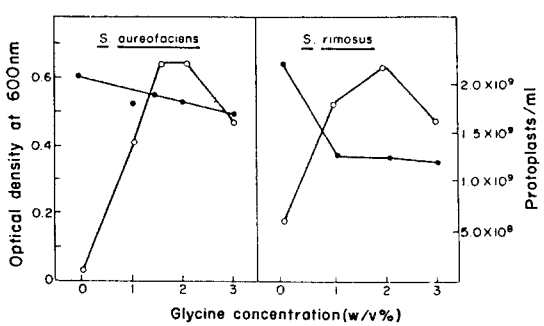


Fig. 1. Effect of glycine concentration of culture medium on growth (●) and protoplast formation (○) in *S. aureofaciens* and *S. rimosus*. Growth was followed by monitoring the absorbance at 600 nm (A_{600}).

regeneration medium were adopted from Okanish's work[8].

General methods for protoplast fusion

For mutagenesis to isolate auxotrophic mutants, spores were treated with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, 1 mg/ml) in TM buffer (0.1 M Tris-(hydroxymethyl) aminoethane and 0.1 M maleic acid, pH 7.0) for 30 min in shaking incubator maintained at 50℃. Auxotrophic mutants were selected by comparing the pattern of colonies on replica made on minimal medium(MM) and complete medium(CM). Procedures to adjust the conditions for preparation of protoplasts and regenera-

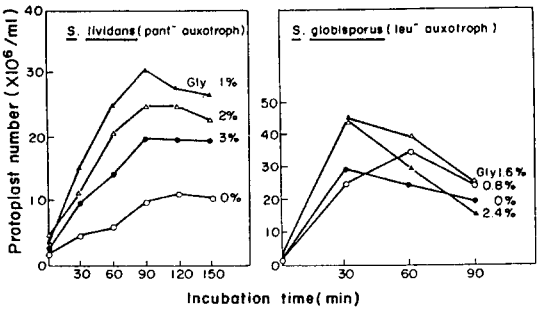


Fig. 2. Effect of lysozyme treatment time on protoplast formation of *S. lividans* and *S. globisporus*.
Protoplasts were quantified by direct counts in a haemacytometer.

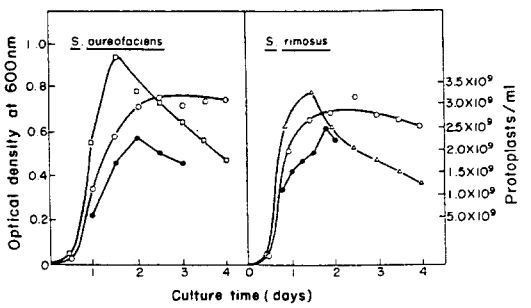


Fig. 3. Cell growth and protoplast formation as a fuction of growth phase in *S. aureofaciens* and *S. rimosus*.
●, the number of protoplast; □, cell growth in 1.5% glycine containing; △, cell growth in 1.0% glycine containing medium; ○, cell growth in 0% glycine medium.

tion were mainly adopted from Baltz[2,3] and Hopwood's manual[9]. For protoplast fusion, approximately the same amounts of parental protoplast suspensions were mixed and incubated with polyethyleneglycol 3350 (PEG 3350) of proper concentration for 30 min at 30℃. The resulting protoplasts were plated to isolate the fusants on fresh minimal R2 medium.

Methods for aniline bioconversion by Streptomyces spp.

After 48 hrs of incubation (180 rpm, 28℃) of each test strain in a fresh screening medium, aniline hydrochloride or acetanilide (500 mg/*l*) was added into the culture broth. After further 5 days incubation, the culture broth was extracted with ethylacetate three times and then the extract was evaporated to dryness. The solid residue was solubilized with

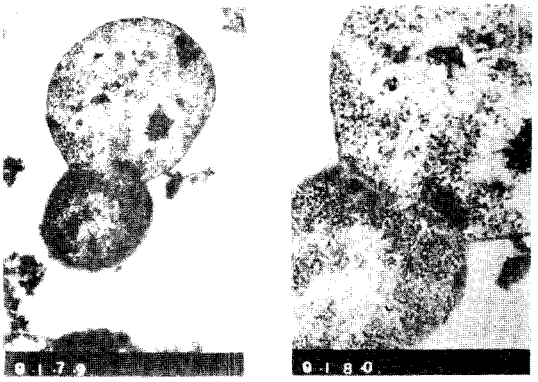


Fig. 4. Electron micrograph of the early stage of cell fusion between *S. lividans* and *S. globisporus*.

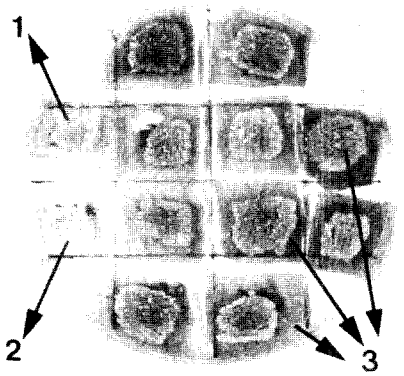


Fig. 5. Selection of mutant on agar plate lost acetanilide p-hydroxylation activity through protoplast formation and regeneration of *S. aureofaciens*.
Colonies regenerated from *S. aureofaciens* protoplast were grown on Theriault's screening agar medium at 29℃ for 5 days and saturated with acetanilide solution (5 mg/m*l*). After 7 day incubation, acetaminophen produced was detected by red color. Reaction of acetaminophen with color reagent (2-nitroso-1-naphthol-4-sulfonic acid, 2.5g/*l* and trichloroacetic acid, 100g/*l*) and $NaNO_2$ (1g/*l*) developed red color. 1, negative control without acetanilide saturation; 2, cured colony; 3, Rest of all blocks are not cured.

50 μ*l* methanol. 10 μ*l* of methanol extract was spotted on thin layer chromatography (TLC) plate and chromatographed in the following solvent system: $CHCl_3/CH_3OH/NH_4OH$ (85:15:1.5). Products were identified by comparison of R_f value. And spot locations were confirmed by quenching of 254 nm induced fluorescence and/or sparying the solution of 1%

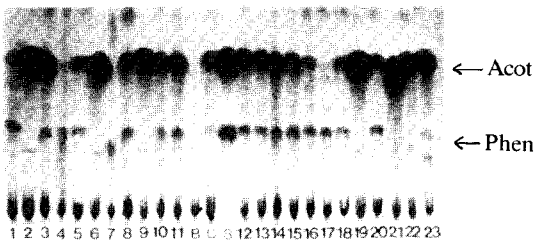


Fig. 6. Detection of acetanilide p-pydroxylation activity in *S. aureofacienc* colonies regenerated from protoplasts using silica gel TLC.

Lane B, negative control, metabolites of *S. aureofaciens* wild type without acetanilide; lane C, control, metabolites of *S. aureofaciens wild type with acetanilide; lane, 2, 6, 9, 19, 22, metabolites of mutants lost p-hydroxylation activity; other lanes, metabolites of strains having p-hydroxylation activity; lane S, standards; Acet, acetanilide; Phen, acetaminophen.*

$K_3Fe(CN)_6$ and $FeCl_3$ (1:1, v/v).

RESULTS AND DISCUSSION

Bioconversion of aniline and acetanilide by Streptomyces

A possible pathway of biotransformation of aniline to acetaminophen is visualized in Scheme 1. In order to obtain acetaminophen directly from aniline through p-hydroxylation and N-acetylation, various fungi and bacteria were screened. Among them, bioconversion activity of *Streptomyces* spp. is summarized in Table I. N-acetylation function on aniline was rather ubiquitous and thus all the tested species except *S. venezuelae* had shown their activities. On the other hand, p-hydroxylation function was only found in *S. aureofaciens, S. griseus* and *S. globisporus*. A newly screened *S. fradiae* NRRL 2702 revealed stronger p-hydroxylation activity than other species in Table I. When *S. fradiae* was grown in Theriault's screening medium, 15-20 mg/*l* of acetaminophen was accumulated at the expense of aniline (500 mg/*l*) in the culture broth.

Interspecific protoplast fusion

For strain improvement, two interspecific protoplast fusion strains were developed between *S. rimosus* (acetylation function) and *S. aureofaciens* (p-hydroxylation function) and also between *S. lividans* (acetylation funtion) and *S. globisporus* (p-hydroxylation function. As genetic markers for protoplast fusion, auxotrophic mutants were induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 1 mg/ml) treatment and they are shown in Table II. For efficient protoplast fusion and cell wall regeneration, various variables such as glycine concentration, cultivation time in medium containing glycine, lysozyme concentration and treatment time length of lysozyme, and condition of ultrasonic fragmentation of mycelia were examined. Parts of these results are shown in Fig. 1, 2 and 3. In a typical experiment, fusion frequency induced between *S. rimosus* (pro⁻, his⁻) and *S. aureofaciens* (ilv⁻) was 8.3×10^{-7} when two strains were treated with 40% polyethyleneglycol 3350 (PEG 3350) for 3 min. But in case of *S. lividans* (pant⁻) and *S. globisporus* (leu⁻), concentration of PEG and fusion frequency were 50% and 1.2×10^{-6} (Fig. 4).

Table II. *Streptomyces* strains used in the fusion experiment

Strain[a]	Genotype[b]	Source
S. aureofaciens	Prototroph	ATCC 10762
SA 1	ile⁻ val⁻ (ilv⁻)	ATCC 10762
SA 2	his⁻	ATCC 10762
SA 3	pur⁻	ATCC 10762
S. rimosus	Prototroph	ATCC 13224
SR 1	pro⁻	ATCC 13224
SR 2	his⁻	ATCC 13224
S. lividans	Prototroph	IFO 12826
SL 1	pant⁻	IFO 12826
SL 2	arg⁻	IFO 12826
S. globisporus	Prototroph	ATCC 1819
SG 1	arg⁻	ATCC 1819
SG 2	leu⁻	ATCC 1819
SG 3	arg⁻ vit⁻	SG 1

[a] All mutants were induced by NTG using spores
[b] Abbreviations: ile, isoleucine; val, valine; his, histidine; pur, purine; pro, proline; arg, arginine; leu, leucine; pant, pantothenic acid; vit, vitamin.

Coloring reaction on agar plates

For detection of p-hydroxylation activity loss of plasmid cured clones on agar plates, a suitable assay method is compulsory. Thus, we devised a sensitive coloring reaction which is applicable to detect acetaminophen formed on a agar plate. Admixture of 2-nitroso-1-naphthol-4-sulfonic acid (250 mg) and

trichloroacetic acid (100 g) in 100 m*l* aqueous solution was dropped on clones of a tested culture which were grown with aniline or acetanilide for 7 days. After diffusion out on the plate, dropping of sodium nitrite (10 mg/m*l*) solution made red color with acetaminophen (Fig. 5).

Plasmid curing and loss of p-hydroxylation activity

S. aureofaciens was treated with sublethal concentration of acriflavine (10 mg/m*l*) at 46℃ for 5 days. The regenerated protoplasts from this mycelia gave most of clones losing their hydroxylation activity (Fig. 6). They grew well on a minimal medium and did not revert even after 5 consecutive transfer for 3 months. The cell regenerated mycelia from protoplasts of *S. aureofaciens* lost their hydroxylation activity at the frequency of 2%.

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