Mutants of *Streptomyces akiyoshiensis* Blocked in 5-Hydroxy-4-oxonorvaline Production

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(Received for publication July 31, 1995)

Streptomyces akiyoshiensis produces a nonprotein amino acid, 5-hydroxy-4-oxonorvaline[1)] (HON; also called antibiotic RI-331[2)]), that is active against tuberculous bacilli[1)] and fungi[3)]. It inhibits homoserine dehydrogenase, thus blocking the biosynthesis of threonine, isoleucine and methionine[2)]. Because these are essential dietary amino acids in animals, HON is potentially useful as a nontoxic antimicrobial drug. In addition, it may have value as a chiral starting material for the synthesis of more complex pharmaceutical agents. Analyses of HON enriched from isotopically labelled precursors have shown that C-1 to C-4 are derived from aspartate, and C-5 from the methyl carbon of acetate[4)], but the biosynthetic pathway is not known. With the aim of better understanding HON biosynthesis, we have examined blocked mutants of the producer.

Materials and Methods

Cultures

Streptomyces akiyoshiensis (ATCC 13480) was obtained from the American Type Culture Collection, Rockville, MD. Wild type and mutant strains were stored at $-20°C$ as spore suspensions in 20% glycerol[5)]. Cultures for HON production were grown in starch-casein (SC) medium, which consisted of (g/liter of distilled water): purified casein (Anachemia, Toronto, Ont) 4.0, soluble starch (BDH) 30, K_2HPO_4 1.75, KH_2PO_4 0.75, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.01, $CaCl_2$ 0.01, $FeSO_4 \cdot 7H_2O$ 4.5×10^{-3}, $ZnCl_2$ 1.8×10^{-4}, $MnCl_2 \cdot 4H_2O$, 4.5×10^{-5}, $CuSO_4 \cdot 5H_2O$ 4.5×10^{-5}, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 4.5×10^{-5}, and H_3BO_3 7.2×10^{-6}. The pH was adjusted to 6.5 before sterilization by autoclaving. A portion (10 μl) of stored spore suspension was used to inoculate each 10 ml of medium in a 125-ml Erlenmeyer flask. Cultures were incubated for 6 days at 27°C and 250 rpm. The HON titre was bioassyed daily, and confirmed by HPLC[6)].

Bioassay

The bioassay agar contained 1.2% (w/v) glucose-Difco yeast nitrogen base without amino acids (GYNB), and was overlayed with 0.4% GYNB agar inoculated with *Saccharomyces cerevisiae* (bakers' yeast[7)]) grown 18 hours in GYNB medium. Assay disks with 30 μl of culture filtrate were applied, and the bioassay culture was incubated for 18~24 hours at 30°C. With similarly seeded agar plates, bioautography of wild-type *S. akiyoshiensis* cultures grown in SC medium and fractionated by paper chromatography in *n*-butanol - acetic acid - water (12 : 3 : 5) showed only a single bioactive zone, which corresponded to HON.

Mutagensis and Mutant Isolation

Suspensions of spores from wild-type *S. akiyoshiensis* grown on maltose - yeast extract - malt extract (MYM) agar[8)] were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine[9)] (NTG) to allow less than 2% survival, and were plated on SC agar to yield single colonies. After 2~3 days at 30°C, the colonies were excised on agar plugs 7 mm in diameter, and incubated for another 5 days as individual plug miniculture. The plugs were transferred to GYNB agar seeded with *S. cerevisiae* for bioassay.

Cosynthesis

Liquid SC medium and SC agar were inoculated with spore suspensions of each strain in pairwise mixtures. Cultures of individual strains served as controls. Production of HON was monitored by bioassay and confirmed by HPLC. Strains exhibiting cosynthesis were examined for the polarity of interactions by exchanging the mycelium and extracellular fluid from pairs of cultures grown individually in liquid SC medium for 48 hours, the age at which the wild type was actively producing HON. The cultures were centrifuged aseptically, and each supernatant solution was syringed through a 0.2 μm pore-size membrane filter before it was combined with the mycelial pellet from its partner strain for further incubation. The exchange cultures were bioassayed and analyzed by HPLC after 24, 48 and 72 hours.

Results and Discussion

Evidence that *S. akiyoshiensis* produced HON as the single product detectable by a convenient bioassay allowed us to screen a large number of mutants for loss of production. Of 4230 colonies examined, 198 gave no inhibition zone. A high proportion of the 198 were bald or unpigmented; they were suspected to have mutations in global regulatory genes, and were not examined further. Those that retained the wild type characteristics of rapid growth, profuse sporulation and dark-red pigmentation through three rounds of single colony isolation were bioassayed again after 6 days growth in SC medium. Only 11 strains still produced $\leq 3\%$ of the wild-type HON titre (12 mM): of these, L021, L086, L138, L156, L159, L174 and L195 gave no activity, and the titres in L016, L127, L141 and L167 were only 0.24, 0.24, 0.36 and 0.33 mM, respectively. HON could not be detected by HPLC analysis of 75% (v/v) aq ethanolic extracts of the mycelia from any of these strains, or from the wild type.

The mutants all grew on minimal medium, and therefore were not blocked in primary metabolic pathways generating HON precursors. The nature of the lesions in secondary metabolism were examined by testing for cosynthesis of HON in pairwise mixtures of the mutants. Strains L127 and L167, mixed individually with L016, L086, L138, L159 or L174, produced the antibiotic in either liquid or agar cultures. Analysis of cosynthetic polarity (Table 1) confirmed the indication from cross-feeding that the cosynthetic mutants could be classified into two groups: group I (L127 and L167) produced HON in response to a substance (or substances) in filtered broth from group II strains (L016, L086, L138, L159 and L174). The non-cosynthetic strains L021, L141, L156 and L195 (group III) neither stimulated group I to produce HON nor responded to group II broths.

Efforts to identify the substance to which group I strains responded were focussed on the broth of strain L138. Quantitative assays based on HON formation by strain L127 showed that activity was associated with a weakly acidic, nonbasic substance that withstood 1 M HCl for 1 hour at 100°C, but not 1 M NaOH. Little activity could be extracted from broth at pH 2 with ethyl acetate, or removed by passage through a C_{18} reverse-phase cartridge. However, activity was retained on charcoal (Darco G-60), and could be eluted with 50% aq acetone. Ultrafiltration indicated a relatively small ($\leq M_r$ 10,000) molecule; this was confirmed by chromatography in water on Sephadex G-10. HPLC (detection at 220 nm) and ^{1}H NMR analysis of bioactive peak fractions identified *N*-acetyl 3,4-dihydroxyphenylalanine which, however, proved not to be involved in HON biosynthesis[10]. The absence of other distinguishable NMR signals in the peak fractions indicated that the quantity of active product was very small, and well below that predicted if it were a biosynthetic intermediate converted to HON in the bioassay.

Because the properties of the active component in L138 broth also differed from those expected in a HON intermediate, alternatives to biosynthetic crossfeeding have been considered for the cosynthesis observed with *S. akiyoshiensis* blocked mutants: one possibility is that HON production depends on the formation of an autoregulator. The properties of the active substance in L138 cultures differ markedly from those of the nonionic, nonpolar A-factor[11] and other[12] γ-butyrolactones known to regulate antibiotic production. They resemble more closely those of B-factor[13] and a putative transcriptional regulator of thienamycin production accumulated by mutant RK-11 of *Streptomyces cattleya*[14]. A second possibility is that the *S. akiyoshiensis* mutants are blocked in the formation of a cofactor for one of the enzymes in HON biosynthesis. Precedents for this include the involvement of vitamin B_{12} in thienamycin biosynthesis[15], and of cosynthetic factor I (7,8-didemethyl-8-hydroxy-5-deazariboflavin) in the biosynthesis of tetracycline by *Streptomyces aureofaciens*[16] and lincomycin by *Streptomyces lincolnensis*[17].

Table 1. Polarity of interactions between cosynthetic blocked mutants*.

Stimulator (filtrate)	Responder (mycelium)						
	L016	L086	L127	L138	L159	L167	L174
L016	-	0	3.1	0	0	0.3	0
L086	0	-	0.3	0	0	0.3	0
L127	0.25	0	-	0	0	0.05	0
L138	0	0	2.8	-	0	0.5	0
L159	0	0	0.9	0	-	0.1	0
L167	0	0	0	0	0	-	0
L174	0	0	2.4	0	0	0.5	-

* Cultures in which the mycelium and filtrate were separated and recombined without exchange, and pairs of wild-type cultures in which the mycelium and filtrate were exchanged were used as controls.

Acknowledgments

We thank the Natural Sciences and Engineering Research Council of Canada for financial support, and the Patrick Lett Fund of the Biology Department, Dalhousie University for a scholarship (to Y.L.). We also thank Dr. R. G. Brown, Biology Department, Dalhousie University for the *S. cerevisiae* strain.

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