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Improvement of Lysine Production by Analog-Sensitive and Auxotroph Mutants of the Acetylene-Utilizing Bacterium *Gordona bronchialis (Rhodococcus bronchialis)*

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ABSTRACT

An acetylene utilizing *Gordona (Rhodococcus) bronchialis* strain, screened for the production of fine chemicals, was found to be capable of producing small amounts of lysine. Attempts to produce amino-acid analog-resistant and/or sensitive mutants and auxotrophs of this strain with increased lysine production were made following UV-irradiation or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) treatment. The bacterium exhibited surprisingly high resistance levels to the aforementioned mutagens which is attributed to highly effective inborn-repair systems. Natural resistance to high levels of *S*-(2-aminoethyl)-L-cysteine (AEC) (2%) was observed, in contrast with D, L-aspartic acid hydroxamate (AAH), L-lysine hydroxamate (LHX) and β-fluoropyruvate (FP). A variety of amino-acid analog-resistant (AAH^r, LHX^r) or analog-sensitive (FP^s) mutants were produced following UV-irradiation or MNNG treatment. Similarly, a large number of auxotrophs (68) of different types were also obtained. From these, one FP^s mono-auxotroph and two poly-auxotrophs (with at least one requirement for the aspartic acid family) showed an

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increased lysine production (~1.8 g/L) comparable (4 g/L) to that found in other bacteria capable of utilizing long-chain hydrocarbons *(1)*.

Index Entries: *Gordona bronchialis*; *Rhodococcus*; lysine production; acetylene utilization; analog resistance; analog sensitivity; auxotrophs; strain improvement.

INTRODUCTION

A well-documented fact in bacteria is that overproduction of metabolites can be induced by the selection of mutants that are resistant to analogs of that metabolite *(2)*. Several mutants of *Brevibacterium* and *Corynebacterium* resistant to the analogs *S*-(2-aminoethyl)-L-cysteine (AEC), DL-aspartic acid hydroxamate (AAH), L-lysine hydroxamate (LHX) or hypersensitive to β-fluoro-pyruvate (FP) have been isolated and used successfully to produce higher yields of lysine *(2–5)*.

Although the microbial metabolism of unsaturated short-chain hydrocarbons has received little attention so far, there is a growing interest in the search for microorganisms capable of degrading gaseous alkenes and alkynes, because of their obvious industrial and environmental importance *(6)*. Following the first report on the biodegradation of acetylene used as the sole carbon and energy source by the anaerobic bacterium *Mycobacterium lacticola (7)*, there are only two other reports, almost 50 yr later, on similar microorganisms, i.e., *Nocardia rhodococcus (8)* and *Rhodococcus* A1 *(9)*. Apart from some basic research on the physiology and biochemistry of such microorganisms (for review *see* ref. *6*), virtually nothing is known about their genetics. A naturally occurring acetylene-utilizing strain (*Rhodococcus* spp.) isolated from soil enriched with acetylene compounds, was found to be capable of producing small amounts of lysine. Thus, we report here on our attempts to increase the levels of production by creating analog-resistant, analog-sensitive and auxotrophic mutants of the bacterium.

MATERIALS AND METHODS

Microorganisms and Media

Naturally occurring acetylene-utilizing bacteria isolated from soil enriched with acetylene *(10)* were screened for amino-acid production. From several strains capable of utilizing acetylene as the sole carbon and energy source, one was found able to produce small amounts of lysine. This ability was also verified by complementation experiments, in which the supernatant of a *R. bronchialis* culture was used successfully to supplement growth of an *E. coli* lys^- auxotroph strain. The strain was identified initially as a *Rhodococcus* spp. and now (Culture Collection, Lab. voor Microbiologie, Gent, Belgium) as *Gordona bronchialis* (renamed recently from *Rhodococcus bronchialis*). It is designated as strain MCM1 throughout this work.

The chemically defined minimal medium (MM) previously described by Bont and Mulder *(11)* was used with minor modifications. Acetylene at 8% (v/v) in liquid media and 10% (v/v) in solid media served as the sole carbon source, whereas $(NH_4)_2SO_4$ at 1.56g/L served as the nitrogen source. The aforementioned medium with the addition of 0.5% yeast extract was the complete medium (CM), and in cases where acetylene was not used in the MM, 1% (v/v) of glycerol was added.

Growth Conditions

All liquid cultures were grown at 28°C under continuous shaking (160 rpm), in specially designed nephlos flasks with silicon seals (based on the Hungate technique), which allowed the insertion of acetylene at the right volumes *(12)*. For quick screening of hundreds of potential mutants tested for lysine production, 4 mL of liquid medium were dispensed into thick-walled culture tubes (Pyrex or Hungate tubes) which were sealed with appropriate oxygen-impermeable rubber seals. Solid media were incubated in anaerobic jars containing 10% (v/v) acetylene at 28°C.

Isolation of Mutants

The effects of various concentrations of the specific inhibitors AEC, AAH, LHX, and FP on the viability and spontaneous mutant production of strain MCM1 were examined on both MM and CM (Fig. 1). Induction of mutants was performed either by UV-irradiation at 12 J/m^2 (as measured by a Jagger meter) or *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG) treatment according to methods described previously *(13,14)*. After preliminary experiments in which various concentrations of MNNG were used, 1000 μg/mL MNNG was established as the best for mutant production and, thereafter, cells from 30mL liquid cultures in late-exponential phase were treated with MNNG in citrate buffer, pH 5.5, for 2h. As *G. bronchialis* belongs to the mycolic acid-containing bacteria, and these usually exhibit significant surface hydrophobicity, the possible clumping of cells was avoided by incubating the treated cells in liquid CM containing 0.5% Tween-80, for 24 h, immediately after mutagenic treatment. At this time, cells were well separated as examined under light microscope. The treated cells were then plated on CM plates containing glycerol as the carbon source. Small colonies appearing after 8 d were transferred to liquid and solid media containing the respective amino-acid analogs, at the concentrations established as the best for mutant isolation (*see* Fig. 1; AAH, LHX, and FP at 0.10, 0.10, and 0.05 μg/mL, respectively).

Auxotrophs were isolated by replica plating large numbers of mutagenized colonies from CM onto MM plates. The auxotrophic requirement was determined by standard methods *(15)*. MM plates were supplemented with amino acids, purines, and pyrimidines at 20 μg/mL, and vitamins at 0.5 μg/mL.

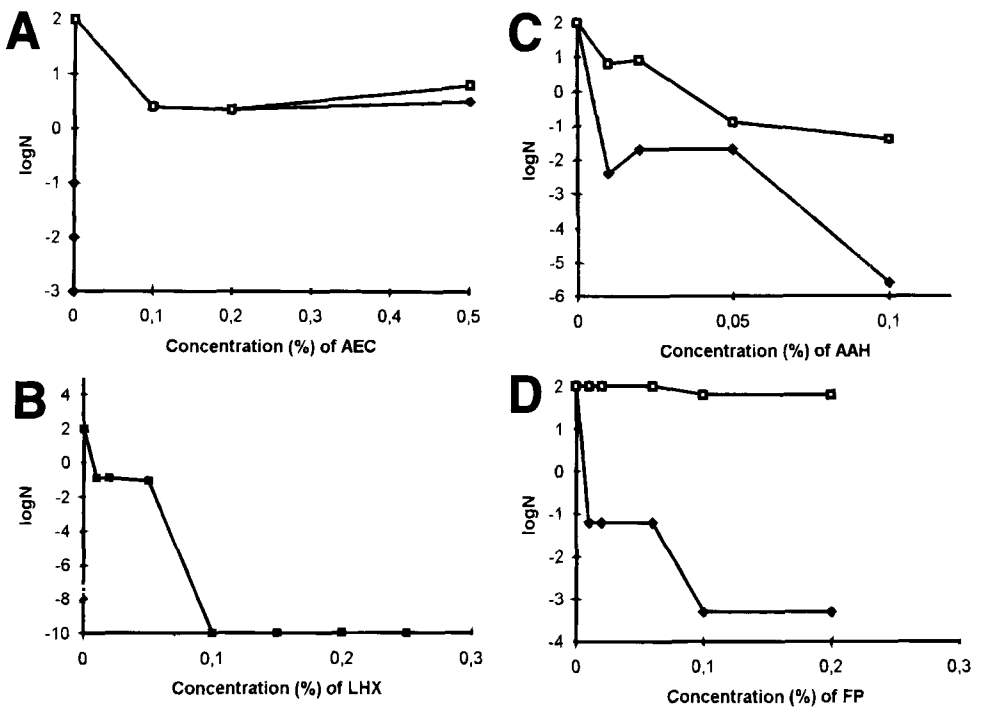

Fig. 1. The effects of various concentrations of amino-acid analogs AEC **(A)**, AAH **(B)**, LHX **(C)**, and FP **(D)** on the viability of the acetylene—utilizing *Gordona bronchialis* strain MCM1 grown on MM and CM. Symbols: A, B, and D— —◆—, MM; —□—, CM. C— —□—, MM, CM.

Lysine Production and Analytical Methods

Screening of potential lysine-producing colonies was performed in small-volume cultures (4 mL) derived from single colonies, following the acid-ninhydrin method *(16)* with minor modifications *(17)*. Quantification of L-lysine was carried out by the Pico Tag method (Waters Ass.), using a compilation HPLC system of Waters. Sigma lysine was used as standard curve.

RESULTS

The effects of AEC, AAH, LHX, and FP on the viability of strain MCM1 grown on MM or CM media are shown in Fig. 1. At the higher concentrations, of all inhibitors used, two morphological types of colonies appeared, i.e., small colonies and normal colonies. However, careful examination of the small colonies showed that in all cases these were phenocopies and were, therefore, excluded from any further studies. AEC has only a minor effect on *G. bronchialis*, (1–2% viability even at 2% concentrations of the inhibitor); and, thus, no further attempts to isolate naturally occurring mutants were made. On the contrary, both AAH and LHX had a

more evident effect, with the latter being the most effective inhibitor. Randomly taken colonies (~200 in each case) from the 0.1% and 1% AAH, as well as the 0.1% LHX plates, were examined for increased lysine production. Only 5 AAH^r (from the 1% AAH plates) and one LHX^r showed a slight increase in lysine production varying from 130–230 mg/L. Inevitably the efforts were concentrated on FP, for which analog it is the sensitive–hypersensitive mutants that may be good producers of lysine. As shown in Fig. 1, the concentration of 0.05% FP appears to be the optimum for isolation of such colonies. Nevertheless, no naturally occurring sensitive or hypersensitive colonies could be isolated at this concentration and it became apparent that induced mutants had to be produced.

The lack of any previous genetic work with this microorganism made it necessary to examine first the effects of UV-irradiation and MNNG treatment on its viability. Thus, a time-course of UV-irradiation was followed and similarly, various concentrations of MNNG and/or time were used (Fig. 2A, B). Although the survival curve indicates that UV-irradiation could be a good tool for mutant production, only a few auxotrophs (0.12%) were isolated, even when over 5000 UV-treated colonies were examined. Here again, two morphological types of colonies (small and normal) were recovered after UV-treatment. In contrast with the situation of amino-acid analog resistance, both types showed 100% viability, and no preference to colony size was found amongst the auxotrophs produced in subsequent subculturings. The auxotrophs isolated had the following requirements: adenine (2), tryptophane (1), asparagine (1), methionine (1), and a polyauxotroph.

The results of MNNG treatment on viability and mutagenicity of the bacterium were somehow surprising. Strain MCM1 shows an extremely high resistance to MNNG although nitrosoguanidine is considered to be a very powerful mutagen, known to give high yields of mutants, at much lower concentrations and/or in lesser time of exposure, as in the case of other bacteria. The 1000 μg/mL MNNG concentration found to have the best mutagenic efficiency here (results excluded for clarity) still allows a great number of cells to survive (i.e., 0.06%). From a total of more than 5000 treated colonies examined, approx 200 colonies originally failed to grow on MM. However, when these colonies were subcultured several times and re-examined, only 68 were found to be stable auxotrophs. Small and normal colonies were observed among surviving colonies, but neither their viability nor their mutagenicity frequencies differed when examined further.

A variety of mutants (68) with different auxotrophic requirements was obtained, indicating a more or less random mutagenic effect of MNNG. Adenine- or leucine-requiring auxotrophs were the most frequent mutants produced. The distribution of the 68 auxotrophic mutants was as follows: ade^- 9, leu^- 9, trp^- 6, aro^- 3, cys^- 3, gua^- 2, pro^- 2, tyr^- 2, arg^- 2, asn^- 2, ala^- 2, his^- 2, met^- 1, asp^- 1, glu^- 1, double mutants 10, poly-auxotrophs

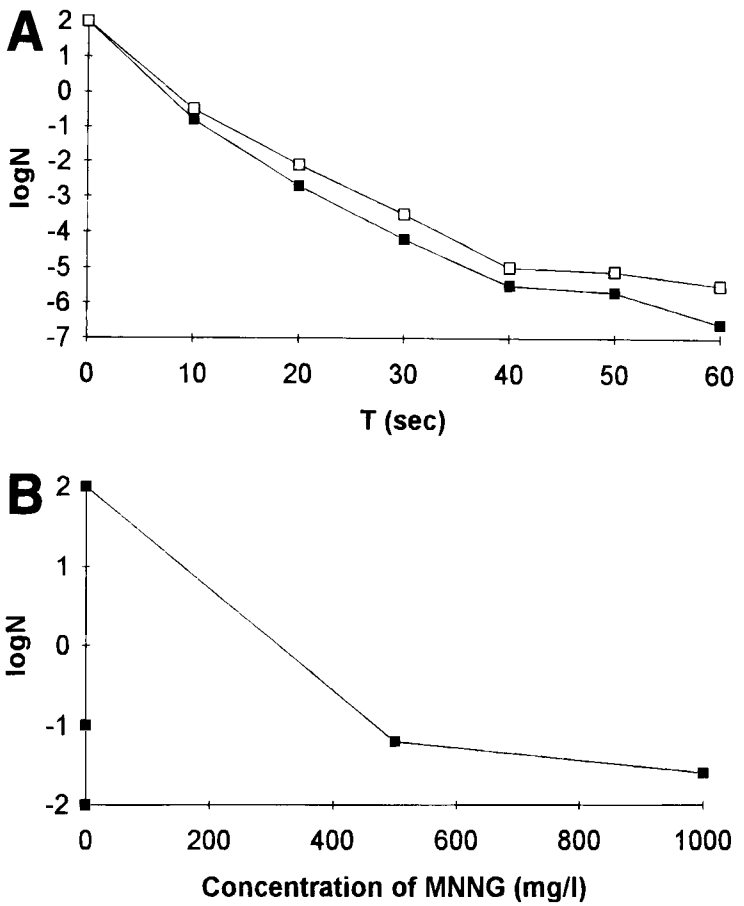

Fig. 2. The effects of UV-irradiation **(A)** and MNNG **(B)** treatment on the viability of the acetylene-utilizing *Gordona bronchialis* strain MCM1. Symbols: —◆—, MM; -□-, CM.

12 (two of which contained at least one auxotrophy for the aspartate-family amino acids). All the aforementioned auxotrophs were tested for AAH, LHX resistance and FP sensitivity, as well as for increased lysine production (see below). Particular emphasis was given to auxotrophs with requirements known from previous reports to be of interest for lysine production (e.g., leucine- and alanine-requiring auxotrophs, (*2,17*)). The two alanine-requiring auxotrophs and 3 out of 9 leucine auxotrophs were found to be FP^s. The FP^s auxotrophs were by far more frequent (1/3 of all the auxotrophs) than the AAH^r and LHX^r ones (5/68 and 1/68, respectively). Auxotrophs with variable genotypes as far as AAH^r, LHX^r and FP^s is concerned were detected. The most interesting of these (e.g., asp^- AAH^r FP^s and leu^- AAH^r FP^s) together with all the auxotrophs of aspartate family were examined carefully for increased lysine production (Table 1).

Table 1
Lysine Production (from 30-mL Cultures) by Auxotrophs, Analog-Resistant and Analog-Sensitive of *Gordona bronchialis* Strain MCM1 (Lysine Production of the Wild-Type Strain Was Less Than 100 mg/L)

Mutant type	No. of isolates tested	Lysine-production (μg/mL)
Auxotrophs[a](A)	13	150–350
Auxotrophs[a](B)	55	100–150
auxotrophs AAH^r	5	100–230
auxotroph LHX^r	1	150–180
prototrophic FP^{s}[b](1)	12	100–300
AAH^r FP^{s}[b](2)	3	400–450
polyauxotrophs. FP^{s}[b](3)	3	700–1800

[a]The 68 auxotrophs mentioned in the text were used here. Class (A) includes the 9 leu^-, 2 ala^- and 2 polyauxotrophs (13 in total). Class (B) includes all the rest (55).

[b]The three classes of FP^s are as follows: (1) sensitive and hypersensitive prototrophs, (2) 2 sensitive auxotrophs and 1 sensitive prototroph, and (3) polyauxotrophs, two of which contain at least one auxotrophy of amino acids within the aspartate family.

Analog FP has been frequently used for the isolation of sensitive mutants with enhanced lysine production (*18,19*). Thus, MNNG-treated cells were examined for sensitivity and hypersensitivity to FP. [As hypersensitive were characterized those mutants which were inhibited even at 10 and/or 100 μmol/L of FP.] From ~4000 treated colonies which were tested (all prototrophic), only a small percentage (1.3%) were found to be FP^s (at 4,000 μmol L^{-1} of FP). The 52 FP^s originally isolated were further tested on eight different concentrations (10, 100, 500, 1000, 1500, 2000, 3000, and 4000 μmol/L) of the analog to determine the limits of their sensitivity. [Mutants inhibited at 10μmol/L of FP were characterized as hypersensitive, whereas those inhibited at 100 μmol/L of FP, or higher concentrations were grouped as sensitive.] Only two were found to be hypersensitive. Nevertheless, as in the case of auxotrophs, all the FP^s mutants were tested for lysine production and the results are presented in Table 1.

DISCUSSION

Although the aim of this work was not directly to study the genetics of *G. bronchialis*, it should be pointed out that the doses of both UV-irradiation or MNNG used by far exceed those needed in other bacteria to induce mutations (*1,3,5*). Moreover, the low frequency of spontaneous-mutant production and the high-reversion rates obtained for mutants produced by either UV-irradiation or MNNG treatment, may be indicative of highly efficient repair systems operating in this organism.

Extracellular lysine production is not restricted to any particular group of microorganisms, though the high yield strains are mostly the species of *Arthrobacter, Corynebacterium*, and *Brevibacterium* (*2,21,22*). A bacterium, therefore, like MCM1 *G. bronchialis*, which can utilize acetylene as its sole carbon and energy source and can produce even small amounts of lysine, is an attractive candidate for the degradation of gaseous alkynes used extensively in the petrochemical industry, with the simultaneous production of a useful chemical. The generally accepted strategy to isolate analogue resistant (AEC^r, AAH^r, or LHX^r) or sensitive (PF^s) mutants which cause a deregulation of amino-acid biosynthetic pathways and hence, the release of increased amounts of amino acids (*17*), was only partially sucessful in our work. AEC, an analog widely used with other bacteria to obtain resistant mutants with increased lysine production (*1,2,23,24*), was ineffective to *G. bronchialis*, because of the high inborn resistance to AEC of the bacterium. Similarly, AAH^r, LHX^r or nonspecific auxotrophs have very little effect on lysine production when compared with the wild type and are in wide contrast with other lysine producing microorganisms (*3,4,25,26*). The specific auxotrophs also (leu^-, ala^-, and polyauxotrophs with at least one requirement concerning the aspartate family) increased lysine production only marginally (2–3-fold). However, as expected, attaining sensitivity to FP-stimulated lysine production of auxotrophs, even if an increase in production was not so considerable (10–18 fold). The 1.8 g/L lysine produced by a polyauxotrophic FP^s mutant of MCM1 is comparable to the 4 g/L produced by *Micrococcus luteus* (*1*) using aliphatic long-chain hydrocarbons and thus, renders this strain potential commercial interest. However, taking into account the large numbers and the variety of mutants examined in this work, it can be concluded that the induction of mutants with high-lysine production yields is a rather rare or slow process with classical-mutagenic procedures. This may be due to the utilization of different biochemical pathways by this acetylene-utilizing bacterium than those used by the commercial-lysine producers (*2,20,23*), or the highly effective repair systems of this strain as exhibited during the mutagenic procedures. Nevertheless, the production of regulatory mutants of the lysine pathway in this work provides useful material for future attempts to genetically manipulate *G. bronchialis* with the corresponding genes of the producer bacteria already cloned in plasmids (*27,28,29*).

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