0273-2289/00/88/0231/$12.75

# Nitrile Hydratase of *Rhodococcus*

*Optimization of Synthesis in Cells and Industrial Applications for Acrylamide Production*

**Tatyana E. Leonova (Pogorelova), Olga B. Astaurova, Ludmila E. Ryabchenko, and Alexander S. Yanenko***

*State Institute of Genetics and Selection of Industrial Microorganisms, 1-st Dorozny proezd, Moscow 113545, Russia, E-mail: yanenko@genetika.ru*

## Abstract

Strain *Rhodococcus rhodochrous* M8 exhibiting high nitrile hydratase (NHase) activity is a promising catalyst for commercial production of acrylamide. With the aim to enhance the biocatalyst activity, regulation of NHase synthesis in this strain has been investigated. The synthesis of NHase and amidase, which also participated in nitriles metabolism in this strain, was induced by isobutyronitrile, acetamide, or urea. At the same time, the addition of glucose, fructose (rapidly metabolizable carbon sources), or ammonium ions to a medium decreased NHase and amidase synthesis. Using Northern blot analysis we demonstrated that these compounds control the expression of NHase at the transcriptional level. The observed effects of glucose and fructose are not related to the decrease in the intracellular concentration of inducer (acetamide), since these compounds cause neither repression nor inhibition of an inducible active system of acetamide uptake. The synthesis of NHase in *R. rhodochrous* M8 is proposed to be subjected to different regulatory circuits, i.e., induction, carbon, and nitrogen catabolite regulation, operating at the transcription level. We have optimized the growth conditions for *R. rhodochrous* M8 on the basis of the obtained data. Application of the improved biocatalyst for acrylamide production allows us to obtain the concentrated (38%) solution of acrylamide.

**Index Entries:** *Rhodococcus rhodochrous*; nitrile hydratase; induction; catabolite repression; nitrogen regulation; acrylamide production.

## Introduction

Nitrile hydratase (NHase) is a microbial enzyme catalyzing hydration of nitriles to the corresponding amides. NHases have been discovered in different microorganisms that can use nitriles as a sole source of carbon

*Author to whom all correspondence and reprint requests should be addressed.

and/or nitrogen. Except NHase, the second enzyme, amidase, participates in nitriles metabolism in these bacteria (Eq. 1).

$$\text{R-CN} + \text{H}_2\text{O} \xrightarrow{\text{NHase}} \text{R-NH}_2 + \text{H}_2\text{O} \xrightarrow{\text{Amidase}} \text{R-COOH} + \text{NH}_3 \quad (1)$$

Recently NHase has attracted much attention as a catalyst for the industrial-scale production of different amides, especially acrylamide *(1)*. Strain *R. rhodochrous* M8 isolated from soil in our laboratory can produce NHase in large amounts, and it is presently used as a biocatalyst for commercial production of acrylamide in Russia *(2)*. We have shown previously that NHase and amidase activities of *R. rhodochrous* M8 greatly increased in the presence of aliphatic nitriles, amides (substrates for these enzymes), and urea *(3)*. We have also found that addition of glucose, fructose (rapidly metabolizable carbon sources), or ammonium ions to a growth medium decreased NHase and amidase activities *(3,4)*.

In this study we investigated the effects of different physiological conditions on synthesis of NHase and amidase and NHase gene expression in *R. rhodochrous* M8. Our results indicate that the synthesis of NHase in *R. rhodochrous* M8 is subjected to different regulatory circuits, i.e., induction, carbon, and nitrogen catabolite regulation, operating at the transcriptional level. The growth conditions for *R. rhodochrous* M8 have been optimized to yield NHase up to 30% of the total cell protein. The application of the improved biocatalyst for production of acrylamide is discussed.

## Materials and Methods

### Strain and Growth Conditions

A wild-type strain *Rhodococcus rhodochrous* M8 *(2)* was isolated by us from soil due to its ability to grow on isobutyronitrile. The strain was grown on a basal salts medium (BSM), containing (g/L): $KH_2PO_4$, 0.5; $K_2HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.005; $CoCl_2 \cdot 6H_2O$, 0.004. Sodium pyruvate (0.03 *M*), glucose (0.05 *M*), fructose (0.05 *M*), or sodium acetate (0.02 *M*) was used as a carbon source. $NH_4Cl$ (0.01 or 0.05 *M*), urea (0.04 *M*), or isobutyronitrile (0.02 *M*) was used as a nitrogen source. The cells were grown for 2–3 d at 30°C with shaking.

### Enzymatic Assays

Nitrile hydratase and amidase activities were assayed in intact cells. NHase activity was assayed in a reaction mixture (1 mL), containing 0.04 mg of cells (dry weight) in 0.01 *M* phosphate buffer, pH 7.6, and 10 mg of acrylonitrile at 20°C for 5 min with shaking. After incubation, the reaction was terminated by the addition of 0.04 mL concentrated HCl. The amount of acrylamide formed in the reaction mixture was determined spectrophotometrically by measurement of acrylamide absorption at 235 nm *(5)* or by gas chromatography. The NHase activity was expressed

as mmol of acrylamide formed per min per milligram of dry weight of cells (U/mg dry wt).

The hydrolytic activity of amidase was determined in a reaction mixture (2 mL) containing 0.2 mg/mL of cells (dry wt) and 10 mg/mL of acetamide in 0.01 *M* phosphate buffer, pH 7.6. The reaction was initiated by the addition of acetamide. The mixture was incubated at 37°C for 30 min. After incubation, the reaction was terminated by the addition of 0.04 mL concentrated HCl and the amount of released ammonia was determined using Nessler's reagent. The amidase activity was expressed as μmol of ammonia formed per min per milligram of dry weight of cells (U/mg dry wt).

### *Polyacrylamide Gel Electrophoresis*

Total protein of the cells disrupted by sonication at 10 kHz for 15 min in 0.1 *M* phosphate buffer (pH 5.8), containing 7.5 m*M* 2-mercaptoethanol and 5 g/L acetamide, was analyzed by 12.5% SDS-PAGE according to ref. *6*. Protein was quantitatively determined by the method of Lowry et al. *(7)*.

### *Northern Blotting*

Bacterial cells disruption with glass beads (420–600 nm, Sigma) and extraction of RNA were done as described previously (*8*). RNA samples were run on a denaturing formaldehyde-agarose gel and transferred to a Hybond-N membrane according to recommendations of the manufacturer (Amersham Corp). A 1.7-kb DNA fragment, containing genes for both α- and β-subunits of NHase from *R. rhodochrous* M8, was labeled with [$^{32}$P]dATP by DNA Labeling Kit (MB1 Fermentas) and used as a specific probe in hybridization experiments. RNA–DNA hybridization was performed according to Maniatis et al. *(9)*.

### *Uptake of [$^{14}$C]Iodacetamide*

Cells were harvested by centrifugation, washed twice with BSM, and suspended in BSM with chloramphenicol (100 mg/mL) to a final concentration of 0.4 mg/mL (dry weight). [$^{14}$C]Iodacetamide (53 mCi/mmol) was added to a final concentration of 0.01 m*M*. Then 0.2-mL aliquots were taken at the fixed time intervals, collected on fiberglass filters (Whatman GF/C), and washed with 2.5 mL ice-cold BSM. The filters were dried and placed in vials containing 7 mL scintillation liquid [5 g 2,5-diphenyloxozole, 0.1 g 2-*p*-phenylene-bis-(5-phenyloxazole)/1 L toluene]. The samples were counted in a "Tractor Analytic Delta300" scintillation counter.

## Results

### *Induction of NHase and Amidase Synthesis*

The growth of *R. rhodochrous* M8 on a medium containing aliphatic nitriles, amides, or urea resulted in significantly increased NHase and amidase activities *(3)*. To determine whether these compounds are the inducers

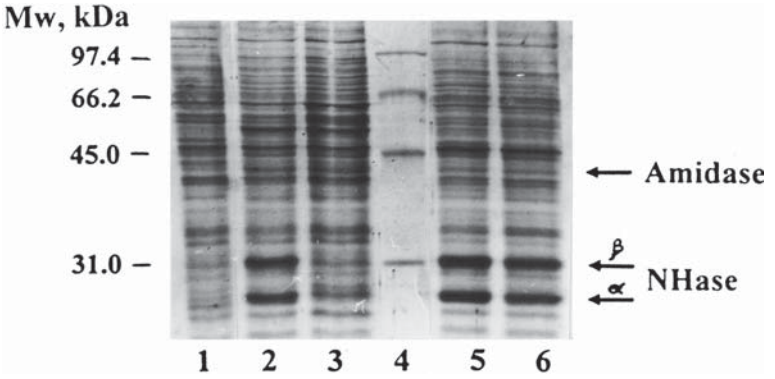


Fig. 1. SDS-PAGE analysis of *R. rhodochrous* M8 total protein. The strain was grown on BSM with sodium pyruvate (0.03 *M*) and $NH_4Cl$ (0.01 *M*) for 48 h, then the following compounds were added: lane 1, without additional compounds; lane 2, urea (0.04 *M*); lane 3, urea (0.04 *M*), but BSM did not contain cobalt ions; lane 5, acetamide (0.04 *M*); lane 6, isobutyronitrile (0.02 *M*), and cultivated for 24 h. Lane 4, molecular mass markers. Each lane contained 40 μg of total protein.

of NHase and amidase synthesis the level of NHase and amidase proteins before and after the addition of isobutyronitrile (substrate for NHase), acetamide (substrate for amidase), or urea was investigated by SDS-PAGE (Fig. 1). Two extensively stained bands (about 26 and 29 kDa), corresponding to α- and β-subunits of NHase, were observed in disrupted cells of *R. rhodochrous* M8 grown in the presence of each of these compounds (Fig. 1, lanes 2, 5, and 6). The intensity of the band (about 42–43 kDa), corresponding to amidase, also increased after the addition of urea, acetamide, or isobutyronitrile (Fig. 1, lanes 2, 5, and 6, respectively). In the absence of these compounds, the bands corresponding to both proteins were almost undetectable (Fig. 1, lane 1). Thus, both NHase and amidase syntheses were induced by isobutyronitrile–substrate for NHase, by acetamide–substrate for amidase and the product of NHase reaction, and by urea.

It should be noted that in the presence of an inducer (nitrile, amide, or urea) NHase and amidase were synthesized in *R. rhodochrous* M8 cells in different amounts. In all cases the yield of NHase was more than 30% of the total protein, whereas the yield of amidase under the same conditions was less than 1% (Fig. 1). Since acrylic acid formed by amidase contaminates the manufactured acrylamide, the low level of amidase in *R. rhodochrous* M8 cells is important for the application of this strain as a catalyst.

To determine the effects of isobutyronitrile, acetamide, or urea on the expression of NHase gene, the level of NHase gene transcript in *R. rhodochrous* M8 cells grown with and without these compounds was analyzed by Northern blotting. Isobutyronitrile, acetamide, or urea were added to cultures grown for 48 h in the absence of inducer. Then the cultures were incubated for 24 h prior to harvesting and RNA extraction. The total mRNA was electrophoresed on a formaldehyde-agarose gel

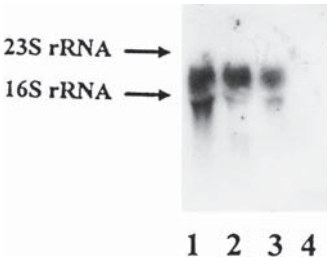


Fig. 2. Northern blot analysis of total RNA isolated from *R. rhodochrous* M8. The strain was grown on BSM with sodium pyruvate (0.03 *M*) and $NH_4Cl$ (0.01 *M*) for 48 h, then the following compounds were added: lane 1, urea (0.04 *M*); lane 2, isobutyronitrile (0.02 *M*); lane 3, acetamide (0.04 *M*); lane 4, without additional compounds, and cultivated for 24 h. Each lane contained 10 μg of total RNA.

and transferred onto a Hybond-N membrane. The blot was probed with a labeled 1.7 kb DNA fragment containing genes of both α- and β-subunits of *R. rhodochrous* M8 NHase. The results shown that after addition of any inducers—isobutyronitrile (Fig. 2, lane 2), acetamide (Fig. 2, lane 3), or urea (Fig. 2, lane 1)—one major transcript (about 2 kb) synthesized. In the absence of these compounds this transcript was not detected (Fig. 2, lane 4). Thus, the significant increase of NHase synthesis in the presence of nitriles, amides, or urea was caused by the activation of NHase gene expression.

The size of the transcript (about 2 kb) corresponded to the size of NHase gene; therefore, NHase and amidase genes were not transcribed as a polycistronic mRNA. Unfortunately, we had no opportunity to study the transcription of amidase gene, since it had not yet been cloned. However, taking into account the simultaneous increase in activities and synthesis of NHase and amidase upon induction *(3,4)*, we assumed the activation of the transcription of amidase gene in the presence of the inducers.

## *Catabolite Repression of NHase Gene Expression*

The addition of glucose or fructose, the rapidly metabolizable carbon sources, to the induced culture of *R. rhodochrous* M8 decreased NHase synthesis *(3)*. To answer the question if the repression of NHase gene expression is the cause of these effects, the level of NHase mRNA before and after the addition of glucose or fructose was investigated by Northern blotting (Fig. 3). Transcription of NHase genes was induced by acetamide (Fig. 3A) or urea (Fig. 3B). The addition of glucose (Fig. 3A, lane 2, Fig. 3B, lane 2) or fructose (Fig. 3A, lane 3, Fig. 3B, lane 3) to the induced culture of *R. rhodochrous* M8 in both cases resulted in a significant, at least fivefold, decrease in the level of NHase gene transcript after 6–8 h of incubation. These results indicated that the decrease in NHase synthesis in the presence of glucose or fructose was due to the repression of transcription of NHase gene. Thus, the expression of NHase is subjected to carbon catabolite repression.

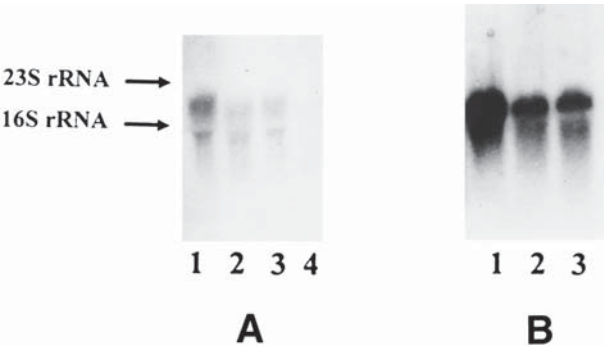


Fig. 3. Northern blot analysis of total RNA isolated from *R. rhodochrous* M8 cultured in the absence or presence of fructose or glucose. **(A)** Total RNA extracted from cultures M8 grown on BSM with sodium pyruvate (0.03 *M*) and $NH_4Cl$ (0.01 *M*) for 48 h, then divided into four portions: a control noninduced culture (lane 4); + acetamide (0.02 *M*) (lane 1); + acetamide and fructose (0.05 *M*) after 60 min cultivation (lane 2); + acetamide and glucose (0.05 *M*) after 60 min cultivation (lane 3) and cultivated for 8 h. Each lane contained 10 μg of total RNA. **(B)** Total RNA extracted from cultures M8 grown on BSM with sodium pyruvate (0.03 *M*) and urea (0.04 *M*) for 48 h, when divided into three portions: a control culture (lane 1); + fructose (0.05 *M*) (lane 2); + glucose (0.05 *M*) (lane 3) and cultivated for 7 h. Each lane contained 3.5 μg of total RNA.

## *The Effects of Glucose or Fructose on Acetamide Uptake in* R. rhodochrous *M8*

The repression of NHase gene expression in the presence of rapidly metabolizable carbon sources may be due to a decrease in the intracellular concentration of an inducer, caused by the inhibition of the inducer uptake by the cell ("inducer exclusion") *(10)*. To clarify this question we investigated the effects of glucose and fructose on uptake of the inducer (acetamide) by *R. rhodochrous* M8 cells.

With the aim of separating the entry process from the processes of cellular metabolism, we used a nonmetabolizable analog acetamide—$^{14}$C-iodacetamide—as a substrate. Since acetamide greatly (by 50–80%) inhibited the uptake of $^{14}$C-iodacetamide in *R. rhodochrous* M8, acetamide and $^{14}$C-iodacetamide entered the cells in the same way.

Uptake of $^{14}$C-iodacetamide was investigated in cells grown on a medium with acetamide alone and acetamide plus glucose or fructose (Fig. 4). The uptake of $^{14}$C-iodacetamide in the noninduced cells was low (Fig. 4). After pregrowth in the presence of acetamide the uptake rate of $^{14}$C-iodacetamide in cells significantly increased (Fig. 4). It allowed us to suggest that *R. rhodochrous* M8 has an inducible system of acetamide uptake. Dinitrophenol (2 m*M*) (an uncoupler of respiration from ATP synthesis) and CCCP (0.1 m*M*) (an ionofor) reduced initial $^{14}$C-iodacetamide uptake rate by acetamide-induced cells from 1.25 nmol/(min · mg protein) to 0.35 nmol/(min · mg protein) or 0.43 nmol/(min · mg protein), respectively. Based on these results we supposed that the acetamide uptake system is an

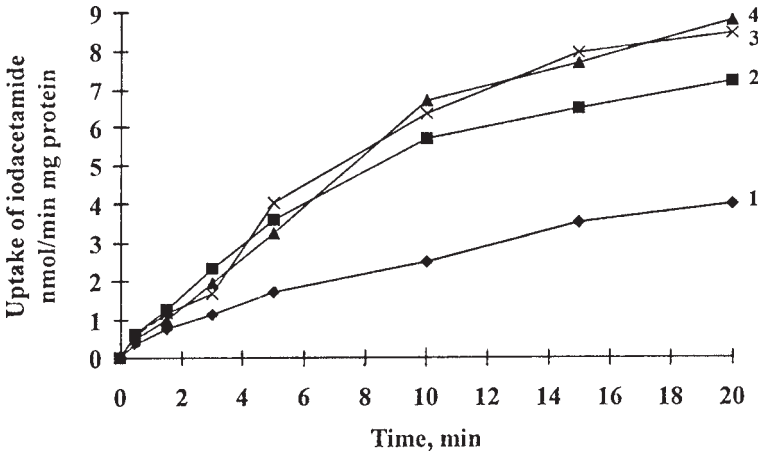


Fig. 4. $^{14}$C-iodacetamide uptake by *R. rhodochrous* M8 cells grown under different physiological conditions. The strain was grown on BSM with sodium pyruvate (0.03 *M*) and $NH_4Cl$ (0.01 *M*), then the culture was divided into four portions: 1, a control, noninduced culture; 2, + acetamide (0.02 *M*); 3, + acetamide and fructose (0.05 *M*) after 60 min; 4, + acetamide and glucose (0.05*M*) after 60 min; and cultivated for 6 h.

active, respiratory energy-dependent system. The residual uptake in cells treated with dinitrophenol or CCCP was probably due to facilitated or simple diffusion.

After pregrowth in the presence of acetamide plus glucose or fructose, the cells took up $^{14}$C-iodacetamide at the same rate as that grown in the acetamide alone (Fig. 4). The absence of effects of glucose or fructose on the acetamide uptake was not connected with the *de novo* synthesis of a transport enzyme (or enzymes) in the reaction mixture, since the determination of the uptake activity was carried out in the presence of cloramphenicol at a concentration sufficient to inhibit the protein synthesis in this bacterium. Therefore, the uptake of acetamide in *R. rhodochrous* M8 was not repressed by glucose or fructose.

The addition of glucose or fructose as inhibitors to the reaction uptake mixture also had no effects on the $^{14}$C-iodacetamide uptake by the induced cells (Fig. 5). It indicated that the acetamide uptake in *R. rhodochrous* M8 was not inhibited by glucose or fructose either.

Thus, the catabolite repression of NHase gene expression by glucose or fructose was not caused by the inhibition or repression of the inducer uptake.

### *The Effect of Ammonium Ions on NHase Gene Expression*

The *R. rhodochrous* M8 NHase activity induced by acetamide or isobutyroamide decreased in the presence of ammonium ions excess in the culture medium, whereas under nitrogen limitation conditions the enzyme activity increased *(3,4)*. To determine if the ammonium effect was due to the repression of NHase gene transcription, the level of NHase gene

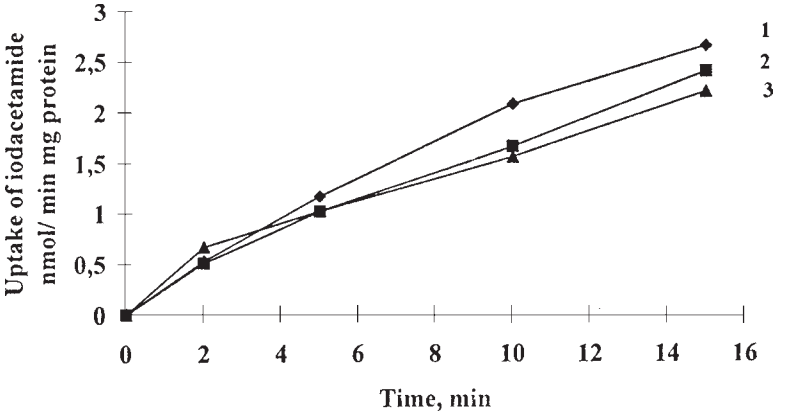


Fig. 5. $^{14}$C-iodacetamide uptake in the absence (1) and presence of fructose (0.05 *M*) (2) or glucose (0.05 *M*) (3) by *R. rhodochrous* M8 cells grown on BSM with sodium pyruvate (0.03 *M*) and $NH_4Cl$ (0.01 *M*) for 48 h and then cultivated with acetamide (0.02 *M*) for 6 h.

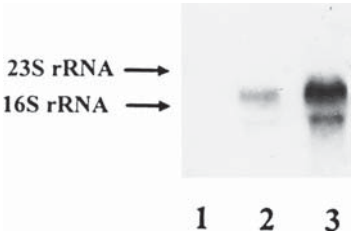


Fig. 6. Northern blot analysis of total RNA isolated from *R. rhodochrous* M8 cultivated with and without ammonium ions (0.05 *M*). Each lane contained 10 μg of total RNA extracted from M8 cultures grown on BSM with isobutyronitrile (0.02 *M*) and sodium acetate (0.02 *M*) for 48 h, then divided into two portions: without addition of ammonium salt (lane 3) and with addition of ammonium salt (0.05 *M*) (lane 2) and cultivated for 1.5 h. Lane 1 contains total RNA from culture of strain M8 grown without an inducer.

transcript under the conditions of excess and limitation of ammonium ions in a medium was examined by Northern blotting (Fig. 6). It can be seen in Fig. 6 that transcription of the NHase genes was induced when cells were grown in the presence of isobutyronitrile (lane 3). The addition of ammonium ions led to a significant decrease in the NHase transcript level after 1.5 h of incubation (lane 2). Thus, the decrease in NHase activity after the ammonium ions addition was due to the repression of NHase gene expression.

### *The Optimization of Conditions for Acrylamide Production by M8 Cells*

The cultural conditions for the growth of *R. rhodochrous* M8 have been optimized to obtain cells with high NHase activity. The strain was grown on BSM, supplemented with urea (7.5 g/L) (as a nitrogen source

and an inducer) and glucose (as a carbon source). With the aim of decreasing the glucose repression, the concentration of glucose was monitored and maintained at a level no more than 5 g/L. The cultivation was carried out at 30°C and pH 7.2–7.4 with shaking during 72 h. Under the above conditions the yield of NHase was up to 30% of the total cell protein. The NHase activity of cells was approx 120 (U/mg dry wt), whereas the amidase activity was only about 0.2 (U/mg dry wt).

These *R. rhodochrous* M8 cells were used as a catalyst for the production of acrylamide. The cells were suspended in water (3 L) to a final concentration of 0.4 g/L. Then acrylonitrile (60 g) was added. The reaction was carried out at 12–20°C with shaking. In the reaction course, the concentration of acrylonitrile in the reaction mixture was determined by gas chromatography and maintained at a concentration below 20 g/L. After a 7-h incubation, the amount of accumulated acrylamide corresponded to 380 g/L. The yield of acrylamide was about 950 g/1 g of the biocatalyst. Since amidase activity in the cells was very low, no formation of acrylic acid was observed in the course of incubation. Thus, direct production of acrylamide at the concentration of 380 g/L can be attained using *R. rhodochrous* M8 cells grown under optimal conditions.

## Discussion

The present study shows that the NHase activity of *R. rhodochrous* M8 cells greatly depends on the presence of inducers and concentrations of glucose, fructose, or ammonium ions.

We demonstrate (Fig. 2) that transcription of the NHase gene is induced in the presence of nitriles (for example, isobutyronitrile), amides (acetamide), or urea. It is interesting that the expression of NHase genes was activated by amides, the products of NHase reaction. The phenomenon that the reaction product is an enzyme inducer is a rare induction mechanism. Usually substrate or related compounds are an inducer of enzyme. However, in the case of various nitrile-utilizing microorganisms (e.g., *R. rhodochrous* J1, *P. chlororaphis* B23) it was found that amides induced synthesis of NHase *(1)*. It is possible that the mechanism of induction of NHase by amide is common for various nitrile-utilizing microorganisms. Further investigation is needed to study this induction mechanism and to clarify the role of different factors in activation of NHase gene transcription by amides.

Our results also demonstrate that the expression of NHase is controlled at the level of transcription by glucose or fructose, the rapidly metabolizable carbon sources (Fig. 3). The repression of NHase gene transcription by these compounds was not caused by the decrease in the intracellular concentration of an inducer, since neither glucose nor fructose repressed or inhibited the uptake of an inducer (acetamide) by the cells (Figs. 3 and 4). We conclude that the synthesis of NHase in *R. rhodochrous* M8 is subjected to carbon catabolite repression. The molecular mechanism

of carbon catabolite repression in high-GS Gram-positive bacteria, such as *Rhodococcus*, is not well understood. It is known that in Gram-negative bacteria (*E. coli*) and low-GC Gram-positive bacteria the protein of glucose-specific PTS plays a major role in the mechanism of catabolite repression *(10)*. However, our preliminary data indicate that the glucose-specific PTS does not function in *R. rhodochrous* M8, like some *Streptomycetes* (high-GC Gram-positive bacteria) *(11)*. We have also shown that cAMP, which participates as effector in the mechanism of catabolite repression in *E. coli (12)*, did not affect the glucose or fructose repression of NHase synthesis (data not shown). In the case of S*treptomycetes* it was also demonstrated that cAMP did not play any role in carbon catabolite repression *(12)*. Thus, we suggest that mechanism of carbon catabolite repression in *Rhodococcus* differs from the mechanism of this repression in *E. coli* and low-GC Gram-positive bacteria. It seems possible that it resembles the mechanism of such regulation in *Streptomycetes* where glucokinase plays a major role *(13)*. Further investigation is needed to clarify this question.

We also observed that the ammonium ions excess decreased the level of NHase gene transcription. Therefore, the NHase expression is controlled at the level of transcription also by nutrient nitrogen. It is not clear how such regulation is realized in this strain, because the molecular mechanism of nitrogen regulation in high-GC Gram-positive bacteria has not been investigated so far.

Previously, we have shown that transcription of NHase gene is also controlled by cobalt ions, which comprise a prosthetic group of this enzyme *(8)*. Thus, the synthesis of NHase in *R. rhodochrous* M8 is subjected to different regulatory circuits, i.e., induction, carbon catabolite repression, nitrogen regulation, and cobalt-dependent regulation, operating at the transcriptional level. We suppose that this complex regulation of NHase gene transcription is required to ensure expression of this gene under different environmental conditions and allows bacteria to switch from one growth condition to another with maximum efficiency. In the case of amidase, we had no opportunity to study the regulation of its gene expression, since its gene has not yet been cloned. However, taking into account that activities of NHase and amidase display simultaneous increase upon induction and decrease after the addition of glucose, fructose, or ammonium ions *(3)*, we suggest that the expression of amidase is also controlled by inducers, nutrient carbon, and nitrogen at the transcriptional level.

## References

1. Nagasawa, T. and Yamada, H. (1990), in *Biocatalysis,* (Abramowicz, D. A., ed.), Van Nostrand reinhold, New York, pp. 277–318.
2. Patent of Russia no. 1731814.
3. Yanenko, A. S., Astaurova, O. B., Gerasimova, T. V., Polykova, I. N., Pogorelova, T. E., and Paukov, V.N. (1995), *Proceeding of the Ninth Symposium on the Actinomycetes*, pp. 139–144.
4. Astaurova, O. B., Pogorelova, T. E., Fomina, O. R., Polyakova, I. N., and Yanenko, A. S. (1991), *Biotechnologya* **N5,** 10–14 (in Russian).

5. Hjort, C. M., Godtfredsen, S. E., and Emborg, C. (1990), *J. Chem. Tech. Biotechnol*. **48,** 217–226.
6. Laemmli, U. K. (1970), *Nature* (London) **227,** 680–685.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
8. Pogorelova, T. E., Ryabchenko, L. E., Sunzov, N. I., and Yanenko, A. S. (1996), *FEMS Microbiol. Lett.* **144,** 191–195.
9. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982), *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
10. Saier, M. H., Jr. (1991), *The New Biologist* **3,** 1137–1147.
11. Titgemeyer, F., Walkenhorst, J., Reizer, J., Stuiver, M. H., Cui, X., and Saier, M. H., Jr. (1995), *Microbiol.* **141,** 51–58.
12. Botsford, J. L. and Harman, J. G. (1992), *Microbiol. Rev.* **56,** 100–122.
13. Angell, S., Lewis, C. G., Buttner, M. J., and Bibb, M. J. (1994), *Mol. Gen. Genet.* **244,** 135–143.