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EXPERIMENTAL ARTICLES

Characterization of the Mutant Recombinant Strain *Pseudomonas putida* IPM-36 Exhibiting Anticipating Growth on a Medium Containing an Inducer of the *cry3A* Gene Expression

N. G. Koretskaya, O. E. Svetoch, O. I. Loseva, and A. P. Dobritsa[1]

State Research Center for Applied Microbiology, Obolensk, Moscow oblast, 142279 Russia

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Abstract—Induction of the expression of the δ-endotoxin gene from *Bacillus thuringiensis* var. *tenebrionis* in the recombinant strain *Pseudomonas putida* IPM-36 negatively affected the viability and the growth rate of the culture. In order to optimize the insecticide production by the recombinant strain, mutant clones exhibiting anticipating growth on an inducer-containing medium were selected and studied. These clones differed in such aspects as the localization of mutations (either in plasmid pBTN11, carrying the *cry3A* gene, or in the chromosome), growth rate, or the level of δ-endotoxin synthesis after induction. Several obtained mutants proved much superior to *P. putida* IPM-36 in their structural and segregation stability, although they were as efficient as the original strain with respect to the production of the insecticide protein Cry3A.

Key words: δ-endotoxin, induction, gene expression, growth rate, genetic stability, *Pseudomonas putida*

Overexpression of cloned genes in recombinant strains disturbs normal functioning of the cell protein-synthesizing system, which leads, as a rule, to reduced viability and growth rate of the cultures, abnormal cell division, and other negative consequences [1–5]. Regulated promoters have been commonly used for the uncoupling of the processes of biomass accumulation and synthesis of the desired product; however, this approach has been only partially successful in preventing the negative consequences of protein overproduction. Thus, with the recombinant strain *Pseudomonas putida* IPM-36 that we constructed, a producer of Cry3A-type δ-endotoxin [5], we failed to fully benefit from the system of regulated gene expression, which offers the advantages of a two-stage fermentation. This strain contains the recombinant plasmid pBTN11 carrying the *cry3A* gene of *Bacillus thuringiensis* var. *tenebrionis*, whose expression is under the control of the following regulatory elements: the *Pm* promoter and the *xylS* gene from the operon of the *meta*-pathway for the degradation of aromatic compounds [6, 7]. The *cry3A* gene expression is induced by the addition of 3-methyl benzoate (3-MB). The high level of protein Cry3A production (0.5–0.6 g/l or 60–70% of total cellular protein) can only be obtained if the *cry3A* gene expression is induced at the initial stage of logarithmic growth [8]. When the inducer was added at the later stages of cultivation, the final yield of protein Cry3A was 1.5- to 2-fold lower. When 3-MB was added during active growth, a dramatic decrease in the growth rate and, under nonselective conditions, an increased number of plasmid-free cells were observed. The earlier was the inducer added to the cultivation medium, the worse were the negative consequences of the induced expression of the *cry3A* gene.

Strain IPM-36 cultures grown on a 3-MB-containing medium accumulate cells harboring mutant plasmids unable to determine the induced synthesis of Cry3A [8]. It could be expected that cells with chromosome or plasmid mutations optimizing the level of the *cry3A* gene expression or enabling the cells to adapt to the overproduction of the alien protein would be characterized by anticipating growth on an inducer-containing medium. With this assumption in mind, we tried to select clones of strain IPM-36 that would show a higher growth rate on a 3-MB-containing medium than the original strain, as well as a high genetic stability and a high level of δ-endotoxin production.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strain *P. putida* BS1356 from the collection of the Laboratory of Plasmid Biology (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences) was used to construct strain *P. putida* IPM-36 [5], whose cells harbor plasmid pBTN11 carrying the

[1]To whom the correspondence should be addressed.

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cry3A gene of *B. thuringiensis* var. *tenebrionis* under the control of the expression-regulating Pm/XylS system. Strain *Escherichia coli* S17-1 [9] contained the *tra* genes of plasmid RP4; these genes can provide for the conjugal transfer of plasmid pBTN11. Plasmid pBTN11 was constructed on the basis of a broad-host-range vector pNM185 [7] and, like this vector, it contains the replicon of plasmid RSF1010 and a determinant for resistance to kanamycin.

Cultivation conditions. The cultures of *P. putida* were grown at 30°C, and those of *E. coli*, at 37°C. LB broth served as a liquid medium [10], and nutrient agar (NA) based on sprat hydrolysate (Dagestan Research Institute of Nutrient Media) served as solid medium. Ampicillin (Amp) and kanamycin (Km) concentrations in solid media were 200 and 50 μg/ml, respectively. In liquid media, antibiotic concentrations were twofold lower. Sodium 3-methyl benzoate was added to a final concentration of 1 mM.

In order to determine δ-endotoxin production, cultures were incubated for 20 h on a shaker at 280 rpm in 50-ml flasks containing 5 ml of the medium supplemented with either Km or Km + 3-MB. When the plasmid stability was evaluated, the cultures were grown on an antibiotic-free medium under the same conditions. The inducer was added to 3-h cultures. Overnight cultures grown in LB with kanamycin served as the inoculum (1%).

When studying the effect of 3-MB on cell growth, the cells of the strains studied were grown in 7 ml of Km-containing LB for 2 h with shaking. Then, 2.5-ml culture samples were transferred into two tubes, and the inducer was added to one of them. The remainder of the culture volume was used to measure the optical density just before 3-MB was added. Incubation continued for another 2 h, and then the optical density of the cultures was measured at 600 nm. The results obtained were used for the calculation of the mean specific growth rate for cultures containing no inducers and for those analyzed 2 h after 3-MB was added. The following equation was used: $t\mu = \ln(X/X_0)$ [11].

Selection for mutants of *P. putida* IPM-36 exhibiting anticipating growth on inducer-containing medium. The mutants were selected in the following three manners. (1) The material of individual colonies grown on kanamycin-containing NA was spread with a loop over an area of 2 cm^2 on the surface of NA containing kanamycin and 3-MB. After incubation for 24 h, the colonies revealed against the background of translucent lawn were picked and studied. (2) 2-μl portions of overnight cultures diluted thousandfold were applied onto the surface of nitrocellulose filters with a pore diameter of 23 μm. The filters were successively incubated on NA containing Km and on NA containing Km + 3-MB (2 h on each medium, five cycles). Then, the cells were washed off the filters with a physiological solution and plated to obtain single colonies on NA containing both kanamycin and 3-MB. One large colony from each filter was analyzed. (3) The material of individual colonies grown on Km-containing NA was spread over an area of 2 cm^2 on NA containing Km + 3-MB. After incubation for 24 h, the cells were harvested with a loop from the regions of heavy growth that contained no mutant colonies selected by the first method. The cells obtained were suspended in 10 μl of LB, and 2 μl of this suspension was applied as a drop onto the surface of Km-containing NA. The cells grown overnight from each drop were suspended in 0.5 ml of LB and 5 μl from each of these suspensions was applied onto an area of 2 cm^2 on NA containing Km and 3-MB. After incubation for 24 h, the cells were taken from those regions of the lawn that contained no single colonies of the fast-growing mutants. Then, the cycle of cell growing on NA with Km and then on NA containing Km + 3-MB was repeated. After five or six such cycles, the cells were plated to obtain single colonies on NA with Km. The clones thus obtained were tested for their ability to show better growth on the inducer-containing medium than the original strain. On the 3-MB-containing medium, the mutants obtained in this way grew more slowly than the mutants isolated by the first method; they failed to form colonies noticeable against the lawn of the original strain.

Isolation and analysis of plasmid DNA. Plasmid DNA isolation, its hydrolysis with restriction endonucleases, and electrophoretic analysis were conducted as described by Maniatis *et al.* [10].

Plasmid elimination. In order to eliminate plasmid pBTN11 and its variants from cells of *P. putida* IPM-36, we used the negative effect of 3-MB on the growth of pseudomonad cells containing plasmid pBTN11, which determines the 3-MB-induced synthesis of protein CryA [5]. The cultures were grown in the presence of 3-MB, plated to obtain single colonies, and kanamycin-sensitive clones were selected. These clones produced no CryA and contained no plasmid DNA.

Plasmid transfer. The cells of *E. coli* S17-1 were transformed with $CaCl_2$-treated plasmid DNA [10]; the transformants obtained served as donor cells for the conjugative plasmid transfer into pseudomonad cells. To mate *E. coli* transformants and pseudomonads, donor and recipient cells were grown together on NA in Petri dishes at 30°C overnight. Selection of both transformants and transconjugants containing plasmid pBTN11 was carried on the Km-containing medium (resistance to kanamycin was the selective trait). On the Amp-containing medium, transconjugants were selected, whereas donor cells were eliminated.

Analysis of δ-endotoxin production. The content of δ-endotoxin in bacterial cells was quantitatively measured by solid-phase immunoenzymatic analysis [5]. The CryA-containing cell inclusions were dissolved for 1 h at 37°C in 100 mM N_2CO_3–HCl buffer, pH 10.5, containing 10 mM dithiothreitol. Total cell protein was measured by the Lowry method [12], using bovine serum albumin as a standard.

Table 1. Characterization of various-type mutants of strain IPM-36, selected for their ability to exhibit anticipating growth on medium containing kanamycin and 3-MB

Mutant type	Mutant number	Localization of mutations	Specific growth rate (μ, h^{-1})*	Amount of δ-endotoxin (mg/ml)*	Proportion of δ-endotoxin in total protein (%)*
I	20	Plasmid	0.69–0.75	≤0.13	≤11
II	56	Chromosome	0.13–0.65	0.16–0.78	23–67
III	4	Plasmid	0.42–0.67	0.18–0.40	15–49
IPM-36	–	Original strain	0.09	0.58	72

* Upon the induction of the *cryA* gene expression.

Table 2. Growth, stability, and productivity of six recombinant strains of *P. putida* upon the induction of the *cryA* gene expression

Strain	Mean specific growth rate on medium with 3-MB (μ, h^{-1})*	Stability		Productivity		
		Segregation (proportion of Km^r clones, %)	Structural (proportion of Ind^- mutants among Km^r clones, %)	Total protein by the Lowry method, mg/ml	Cry3A protein, mg/ml	Cry3A proportion in total protein, %
N26p1 (pBTN11)	0.34	76	<3	1.12	0.59	53
N45p1 (pBTN11)	0.20	70	5	1.2	0.62	52
N56p1 (pBTN11)	0.20	68	<3	0.96	0.64	66.5
N68p1 (pBTN11)	0.13	70	3	1.1	0.68	64
BS1356 (pBTN11-72)	0.44	88	>3	1.0	0.41	41
IPM-36–BS1356 (pBTN11)	0.09	34	20	0.84	0.58	69

Note: The mean values for two or three independent experiments are given.

* In the first 2 h after the inducer addition.

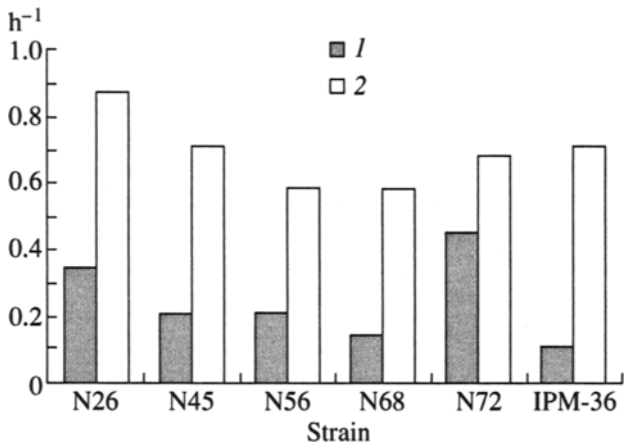
RESULTS AND DISCUSSION

On a solid nutrient medium containing no inducer, the strain *P. putida* BS1356 and the derivative strain IPM-36 differed little in growth. In 24 h, both strains formed on NA a densely populated lawn in regions where a great number of cells had been plated. Upon plating diluted cultures, single colonies of about 1.2 to 1.3 mm in diameter appeared. On the medium with kanamycin, the IPM-36 colonies had the same size: the cells of this strain contain pBTN11 plasmid conferring the resistance to kanamycin.

However, after 24 h of incubation on NA with both kanamycin and 3-MB, the colonies of strain IPM-36 were extremely small (less than 0.2 mm in diameter), whereas the cell lawn was translucent. However, on this lawn, colonies formed by cell variants exhibiting a higher growth rate were revealed at a frequency of 10^{-5}. Cells from these colonies exhibited a stable capacity for anticipating growth on the inducer-containing medium; they were evidently mutant with respect to this trait. When plated on the medium containing both Km and 3-MB, these cells formed colonies of 0.2 to 1.2 mm in diameter. On the inducer-free medium, they did not differ in size from the colonies formed by the original strain IPM-36.

We studied 80 independent clones capable of anticipating growth on the inducer-containing medium; these clones were obtained using three different techniques for selection of *P. putida* mutants (see MATERIALS AND METHODS). These mutant clones were divided into three groups based on the following properties: specific growth rate during the first 2 h after the inducer addition, localization of the mutations, and the level of δ-endotoxin production (Table 1).

The first-type mutants (20 clones) grew equally well on the media containing or lacking the inducer. These mutants were either incapable of δ-endotoxin production or synthesized small amounts of this protein (no more than 11% of total cellular protein). The first-type colonies contained mutant plasmids able to confer the phenotype described above when transferred to *P. putida* BS1356. DNA electrophoresis revealed that these plasmids lacked relatively large fragments of pBTN11 genome. In plasmid pBTN11-10, almost the entire *cryA* gene had been deleted (the strain carrying this plasmid produced no insecticide protein). Another plasmid, pBTN11-15, lacked the DNA fragment con-



Mean specific growth rate of strain IPM-36 and its mutants (*1*) in the first 2 h after 3-MB addition and (*2*) during the same period of cultivation in the absence of the inducer.

taining the *Pm* promoter, whereas the *Hind*III fragment carrying the *cryA* gene remained intact. The other plasmids seemed to carry point mutations or microdeletions.

The second-type mutants (56 clones) were sensitive to the inducer; however, their growth rate on 3-MB-containing medium was higher than that of the original strain *P. putida* IPM-36, and they were superior to the first-group mutant in δ-endotoxin production. In various second-type mutants, the content of Cry3A protein varied from 23 to 67% of the total protein of induced cells. Chromosomal mutations rather than plasmid ones were responsible for the phenotype of the second-type mutants, since their plasmids did not confer the above-described phenotype on the recipient cells when transferred to the strain *P. putida* BS1356 by conjugation. This conclusion was also supported by experiments in which plasmid elimination from cells of the second-type mutants followed by a transfer of plasmid pBTN11 into the cured cells caused no changes in the phenotype.

Four mutants, designated N23, N67, N71, and N72, belonged to the third type. They resembled second-type mutants in such parameters as the growth rate on 3-MB-containing medium and the level of δ-endotoxin production, but, unlike second-type mutants, they carried plasmid mutations. The cells of *P. putida* BS1356 acquired the phenotype of third-type cells when plasmids from third-type clones were transferred into them.

In all studied strains incubated on medium containing no inducer, the level of CryA production did not exceed 11% of the total cellular protein.

We studied in detail several second- and third-type mutants, which were not only superior to the original strain in the growth rate on 3-MB-containing medium but also showed a high level of CryA production. First-type mutants had lost their capacity for induced δ-endotoxin synthesis and were of no interest as producers of the insecticide protein.

Four strains—N26, N45, N56, and N68—were the most promising among the large group of second-type mutants. To preclude the possibility of plasmid-localized mutations in the selected variants, the resident plasmids of these strains were replaced with a plasmid isolated from *P. putida* IPM-36. Transconjugants obtained by mating the mutant strains cured from plasmids with the donor *E. coli* S17-1 strains (pBTN11) are characterized in Table 2 (designations for the cured strains differ from those of the original strains by *pl* endings).

The mutant strains were similar to strain IPM-36 in the yield of Cry3A on medium containing kanamycin and the inducer. However, the mutant strains were superior to the original strains in another parameter, genetic stability, which is important for the recombinant producers. Thus, after incubation for 20 h on non-selective medium (with the inducer added to 3-h cultures), the number of cells that retained the plasmid was twice higher in the mutant strains than in the original strain IPM-36 (Table 2).

We have previously reported [8] that the induced transcription of the *cryA* gene from the *Pm* promoter in strain IPM-36 affects the cells negatively and predetermines the accumulation of type-I, or Ind⁻, mutants harboring altered plasmids that have lost their ability to provide for the 3-MB-induced synthesis of protein CryA. After 20 h of growth with the inducer added to 3-h cultures, about 20% of the IPM-36 cells that retained the ability to form kanamycin-resistant colonies on NA were incapable of determining the 3-MB-induced synthesis of δ-endotoxin. The number of such cells in cultures of type-II mutants incubated under the same conditions did not exceed 3 to 5% (Table 2). Hence, not only the maintenance stability, but also the structural stability of the plasmid was much higher in these cells than in those of strain IPM-36.

In cells of all four type-III mutants (with plasmid localization of the mutations), the level of induced synthesis of the insecticide protein was lower than in the IPM-36 cells. Such a phenotype should be expected in the case if mutations occur in the sequences of the *Pm* promoter or the *xylS* gene. These mutations do not preclude the possibility of the induced transcription of the *cryA* gene, but they might reduce its efficacy, e.g., by interfering with the formation of a complex between *Pm* and the activated protein XylS. It may well be that, at some stages of culture growth after induction, the copy number of the mutant plasmids decreased as compared to that of the original plasmid pBTN11.

Among type-III mutants, strain N72 synthesized the largest amount of protein CryA (0.40 mg/ml, or 49% of the total cellular protein). To preclude the possibility of some chromosome mutations causing an additional effect, the plasmid of this strain, referred to as pBTN11-72, was transferred into strain *P. putida*

BS1356 characterized in Table 2. As it is seen, induced expression of the *cryA* gene localized in pBTN11-72 had a much less negative effect on the strain growth than that observed with strain IPM-36. During the first 2 h after the inducer was added, the specific growth rates of the two strains compared were 0.44 and 0.09 h^{-1}, respectively. In this parameter, strain BS1356 (pBTN11-72) was even superior to the type-II mutant strains characterized in Table 2. The strain harboring the mutant plasmid was significantly more stable after induction and, like type-II mutant strains, it produced a somewhat larger amount of total cellular protein than strain IPM-36 (Table 2). Although during cultivation in a tube with LB medium the level of CryA synthesis in strain BS1356 (pBTN11-72) was lower than in the original strain, it should be kept in mind that the advantages of the strain harboring the mutant plasmid may be realized well under conditions of currently developed technologies for bacterial fermentation.

Note that during incubation on 3-MB-free medium, the growth rates of strains N45pl, N56pl, and N68pl harboring plasmid pBTN11 ranged from 0.58 to 0.71 h^{-1}, thus being either lower or close to the growth rate of the noninduced strain IPM-36 (0.70 h^{-1}). Hence, it is unlikely that the higher growth rate of the mutants on the inducer-containing medium resulted only from their higher metabolic activity. The mutations that occur in the genome of these strains seem to partially compensate for the deficiency in certain components of the cell protein-synthesizing system that are required during the overproduction of the insecticide protein. The specific growth rate of the strain N26pl (pBTN11) on the inducer-free medium (0.87 h^{-1}) was somewhat higher than that of strain IPM-36. The mutations in N26pl possibly affect the synthesis of products with a broader role in the cell activity than the mutations observed in the other variants studied.

A regular trend was characteristic of the mutant strains: the deviation of their mean specific growth rate from that of the original strain IPM-36 depended on the method used for selection of the mutant strain (figure). Thus, the highest growth rate on the inducer-free medium was exhibited by mutant N26 selected by several cycles of incubation on filters (the second method). Most of the other mutants selected by the same method also exhibited a high growth rate.

Noninduced cultures of the mutant strains N56 and N68 selected by the third method showed a somewhat lower growth rate than that of the original strain IPM-36. Hence, in the induced cultures of these strains, the growth rates of plasmid-containing and plasmid-lacking cells were, respectively, higher and lower than that of the analogous cells in strain IPM-36. The relatively high stability of strains N56pl (pBTN11) and N68pl (pBTN11) should be to a certain degree attributed to this fact.

The noninduced cultures of the mutants N45 and N72 selected by the first method (one-stage selection without successive reinoculations) resembled the original strain IPM-36 in their growth rate (figure).

Thus, we have demonstrated in this study that the selection of mutants exhibiting anticipating growth on the inducer-containing medium can yield clones of *P. putida* IPM-36 that are more promising producers of protein Cry3A than the original strain. We believe that an analysis of a great number of mutant clones selected by different methods and combining in a single strain of several mutations conducive to the overproduction of the alien protein should result in further optimization of the Cry3A production. The same approach may be used to improve the properties of other producers in which the induction of intense synthesis of the desired product affects negatively cell growth and division.

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