There are two basic problems associated with the introduction of genetically engineered microorganisms (GEMs) into the environment. The first, ecological, problem is related to possible detrimental effects of recombinant microorganisms and the products of their genes on native ecosystems [1], whereas the second problem is due to the possible influence of the environment on the microbial metabolism and, thus, on the expression of recombinant genes.

It is known that surrounding conditions can affect microbial metabolism through the repression or activation of some operons of chromosomes or plasmids. Such regulation may occur at the level of the biosynthesis of regulatory proteins and thus promote natural selection in the direction of the adaptation of a bacterial population to new environmental conditions [2].

Earlier [3, 4], we showed that the recombinant Escherichia coli Z905 strain containing a plasmid bearing the lux-genes of luminous bacteria and the ampicillin resistance gene can survive in various aquatic ecosystems; however, the expression of the cloned lux-genes may change.

The present work was undertaken to study the effect of two environmental factors, nutrient concentration and the presence of a selective antibiotic, on the mechanism of the regulation of the catabolite-dependent lux-operon cloned in recombinant E. coli cells.

EXPERIMENTAL ARTICLES

Effect of Environmental Factors on the Expression of the Catabolite-Dependent lux-Operon Borne by a Recombinant Plasmid

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Received February 19, 1997

Abstract—Expression of the lux-genes cloned in the recombinant plasmid pPHL7 (Ap'Lux+) in Escherichia coli Z905 cells was studied in various environments, including model aquatic ecosystems. Expression of the lux-genes strongly depended on the nutritional status of the medium. In particular, the cultivation of cells in nutrient-rich medium favored the maintenance of the initial level of expression of the lux-operon, whereas nutrient limitation induced recombinant cell variants with an impaired control of the catabolite-dependent lux-operon. On the other hand, long-term laboratory cultivation of the recombinant strain in nutrient-deficient media or its long-term life in model aquatic ecosystems led to the accumulation of cells with a stringent control on the cloned lux-genes in the bacterial population. The presence of the selective factor (ampicillin) in the medium had no significant effect on the expression of the lux-operon.

Key words: Escherichia coli, recombinant plasmid, lux-operon, regulation of expression, catabolite repression, introduction into model ecosystems, environmental factors.

MATERIALS AND METHODS

Experiments were performed with the Escherichia coli Z905 (hsdR+, hsdM+, gal+, met+, supE, recA, tet+) strain carrying the recombinant plasmid pPHL7 (Ap'Lux+) and its variants isolated from aquatic ecosystems with different lengths of trophic chains. The lux-operon was cloned on plasmid under the control of the lac-promoter [5].

The recombinant strain and its variants were cultivated in complete or tenfold diluted medium M9 [6] supplemented with 50 μg/ml ampicillin as a selective factor.

Factors affecting the expression of the cloned lux-operon were studied by culturing E. coli cells on the above agar media (ten culture transfers). Each of the ten subcultures was plated to obtain colonies. The luminescence of colonies was estimated visually in the dark. The number of colonies selected for analysis ranged from 30 (if their luminescence varied insignificantly) to 70–100 (if their luminescence varied significantly).

Expression of the lux-operon was studied by cultivating strains in test tubes with 10 ml of complete medium. The catabolite repression of luminescence was estimated by adding 1 ml of 10% glucose solution at the exponential phase.

The optical density of bacterial suspensions was measured at 540 nm on a KFK-2 photocolorimeter. The
luminescence of bacterial suspensions was estimated with a luminometer [4, 7].

Plasmid DNA was isolated by the alkaline method [8]. Electrophoretic analysis of plasmid DNA was performed in 0.8% agarose gel.

**RESULTS AND DISCUSSION**

Adaptation of microorganisms to the environment implies, first of all, modification of their metabolism regulation. In this work, adaptation mechanisms were studied in the recombinant *E. coli Z905* strain.
The operation of the lux-operon is under the stringent control of the repressor protein and catabolite activator protein (CAP). Synthesis of these regulators depends on environmental conditions. It is known that biosynthetic processes are largely influenced by the nutritional status of the environment. In our case, the expression of the lux-operon might also be dependent on the presence of ampicillin in the medium, since it had been cloned together with the ampicillin-resistance gene encoding β-lactamase.

Expression of the cloned lux-genes was studied by subculturing *E. coli* Z905 on agar media with different concentrations of nutrients and with or without ampicillin.

During the cultivation of the recombinant strain in complete medium without ampicillin, the initial level of expression of the lux-operon was largely retained (Figs. 1a and 2a), so that cells with impaired regulation (i.e., whose luminescence either had no latent period or was insensitive to catabolite repression with glucose) appeared only in the fourth or fifth bacterial subcultures (Fig. 1b). A considerable decrease in the number of such cells in the tenth subculture indicated their inability to compete with cells capable of retaining or even enhancing control over the lux-operon (Fig. 1c).

In complete ampicillin-containing medium, recombinant cells with impaired control over the lux-operon were found to occur in the second subculture (Fig. 2b), which can be accounted for by the selective pressure of ampicillin. Indeed, to survive in the presence of this antibiotic, cells must enhance the synthesis of β-lactamase [9]; i.e., cells with decreased binding of the protein repressor to the promoter region of the cloned lux-operon will have an advantage. However, during subculturing, the number of recombinant cells with impaired control over the lux-operon gradually decreased, so that cells with the initial level of control over the lux-operon (Fig. 2b) and with a medium level of luminescence were dominant in the tenth subculture grown in complete medium with ampicillin (Table 1).

In the case of the tenfold-diluted nutrient medium, the lux-operon was similarly expressed under selective and nonselective conditions (Figs. 2c and 2d); however, cells with enhanced control over the lux-operon accumulated faster in the presence of ampicillin than in its absence. It could be suggested that ampicillin, as a selective factor, affected the bacterial population by favoring the selection of recombinant cells with initial or enhanced control over the plasmid-borne lux-genes (Fig. 2d).

Irrespective of the nutritional status of the medium, cells dominant in the tenth subculture of *E. coli* Z905 had stronger control over the expression of the lux-genes and, hence, a decreased level of luminescence (Fig. 1c and Table 1). The only difference between rich and diluted media was that, in the latter case, cells with stronger control appeared as soon as in the first subcultures. The considerable reduction of cell luminescence in media without ampicillin could be accounted for not only by the enhancement of lux-operon control, but also by a decrease in the copy number of the recombinant plasmid. As follows from Fig. 3, the recombinant cells in the tenth subculture with both impaired and enhanced control over the lux-operon displayed a reduced copy number of the recombinant plasmid; this phenomenon was especially pronounced in the absence of ampicillin.

<table>
<thead>
<tr>
<th>E. coli Z905 variant</th>
<th>Cell luminescence, µA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variants isolated from the 10th subculture grown on complete medium without ampicillin</td>
<td>10^{4}–1</td>
</tr>
<tr>
<td>complete medium with ampicillin</td>
<td>10^{2}–10^{-1}</td>
</tr>
<tr>
<td>tenfold diluted medium without ampicillin</td>
<td>10^{2}–10^{-1}</td>
</tr>
<tr>
<td>tenfold diluted medium with ampicillin</td>
<td>10^{3}–10^{-1}</td>
</tr>
<tr>
<td>Variants isolated 1 month after introduction</td>
<td>1–10</td>
</tr>
<tr>
<td>6 months after introduction</td>
<td>10^{2}–1</td>
</tr>
<tr>
<td>15 months after introduction</td>
<td>10^{4}–10^{-1}</td>
</tr>
<tr>
<td>2–3 years after introduction</td>
<td>≤10^{-4}</td>
</tr>
</tbody>
</table>

![Fig. 3. Electrophoretic pattern of plasmid DNA. Lanes: (1) *E. coli* Z905 variant with enhanced regulation of the lux-operon isolated from complete medium; (2) variant with enhanced regulation of the lux-operon isolated from tenfold diluted medium; (3) variant with impaired regulation of the lux-operon isolated from complete medium; (4) variant with impaired regulation of the lux-operon isolated from tenfold diluted medium; (5) *E. coli* Z905 variant with normal regulation of the lux-operon.](image)
Thus, the diminished luminescence of the recombinant E. coli Z905 cells could be associated with both enhanced control over the lux-operon and the decreased copy number of the recombinant plasmid. Alterations in the growth conditions (e.g., as a result of the introduction of the strain into the environment), may bring about heterogeneity of a bacterial population in a manner dependent on plasmid structure as well as on biotic and abiotic environmental factors.

In experiments with the recombinant E. coli Z905 strain introduced into various aquatic model ecosystems (MESs) (Table 2), virtually all of the variants reisolated displayed impaired control over the cloned lux-genes (Table 1). According to the degree of control over the cloned lux-genes, the cell variants reisolated from MESs can be divided into three groups (Fig. 4 and Table 1): (i) variants III-13 and II-58 with the initial level of regulation of the lux-operon and a level of luminescence reduced by 1–2 orders of magnitude, which were isolated 1–3 months after the introduction of the strain; (ii) variants I-1 and II-55 with impaired control over the lux-operon and a level of constitutive luminescence reduced by 2–4 orders of magnitude, which were isolated 6–15 months after the introduction of the strain; and (iii) variants I-85 and II-76 with enhanced control over the lux-operon and a considerably reduced level of luminescence, which were isolated 2–3 years after the introduction of the strain.

Analysis of plasmid DNA (Fig. 5) showed that the variants of E. coli Z905 with a low level of luminescence typically had a reduced copy number of the recombinant plasmid. Weak luminescence was presumably due to the insufficient intracellular concentration of the lux-operon inducer or of the aldehyde substrate of the luminescence reaction [7]. Thus, in all three of the MESs studied, variants with enhanced control over the lux-genes gained a selective advantage over other variants.

To conclude, the level of expression of the luminescence system genes cloned in the recombinant E. coli Z905 cells considerably depends on the environmental conditions; namely, high concentrations of nutrients and the presence of a selective factor (ampicillin) favor the maintenance of the initial level of control over the lux-operon, whereas nutrient limitation induces the rapid accumulation in the bacterial population of variants with impaired control over the catabolite-dependent lux-operon, which are then replaced by variants

| Table 2. Characteristics of model ecosystems used for introduction of the recombinant E. coli Z905 strain |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| MES | Trophic chain composition | E. coli Z905, cells/ml | Bacteria, cells/ml | Algae, cells/ml | Total PO₄³⁻, mg/ml | Total N, g/l | COD*, mg O₂/l |
|-----|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| I   | Protozoans | 0.12 × 10² ± 8 | 2.9 × 10⁴ ± 0.6 × 10³ | 2.5 × 10⁶ ± 0.7 × 10⁴ | 0.50 ± 0.03 | 1.50 ± 0.04 | 11.9 ± 0.4 |
| II  | Protozoans, daphnias | 1.0 × 10² ± 31 | 2.5 × 10⁴ ± 0.2 × 10³ | 5.5 × 10⁵ ± 0.6 × 10³ | 1.85 ± 0.07 | 1.85 ± 0.08 | 10.6 ± 0.4 |
| III | Protozoans, daphnias, fish | 2.9 × 10³ ± 0.3 × 10² | 1.7 × 10⁵ ± 0.3 × 10⁴ | 6.4 × 10⁶ ± 0.3 × 10⁴ | 1.70 ± 0.06 | 3.00 ± 0.15 | 13.5 ± 0.5 |

* COD stands for chemical oxygen demand (an index of the overall soluble organic matter).
with a reduced level of luminescence. The effects of environmental factors on the expression of the lux-operon in the recombinant *E. coli* Z905 cells living under laboratory conditions or in model ecosystems are similar.

**ACKNOWLEDGMENTS**

This work was supported by the Russian Foundation for Basic Research (project no. 97-04-49988).

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