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Letter to the Editor

Heterologous Gene Expression in an *Escherichia coli* Population Under Starvation Stress Conditions

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Abstract. A novel system to study the evolution of transcription signals in heterologous systems under selective starvation conditions is described. It is based on the plasmid-mediated transfer of *his* biosynthetic genes from *Azospirillum brasilense* into a heterologous *Escherichia coli* mutant population lacking histidine biosynthetic ability. We show that under highly selective stressful conditions, genetic changes in the donor plasmid lead to mutated sequences that are efficiently recognized as promoters by the *E. coli* RNA polymerase.

Key words: Starvation conditions — Promoter-creating mutations — Heterologous gene transfer — Metabolic evolution — Adaptive mutations

Introduction

''Directed evolution'' experiments in which populations of microorganisms are challenged under stress conditions with novel substrates have shown that the development of new catabolic activities is often due to the recruitment of preexisting enzymes following regulatory mutations (Lin et al. 1976; Mortlock and Gallo 1992; Hall and Hauer 1993). As demonstrated by the rapid interspecies dispersal of biodegradative and antibiotic-resistance genes, the capture of xenologous sequences carried by plasmids of different host ranges can also lead to the accretion of catabolic abilities. As suggested by the incongruencies between deep phylogenies based on different macromolecules, which have been explained by horizontal gene transfer (Gogarten et al. 1996) and even cell fusion events (Sogin 1991; Golding and Gupta 1995), the acquisition of metabolic activities from donor cells to heterologous recipients may have taken place since Archaean times.

How can the newly acquired genes be brought into the preexisting regulatory system of the host organism? Although it is reasonable to assume that mutational adjustment of preexisting promoter sequences would take place, this possibility has not been addressed. This issue can be analyzed under laboratory conditions, by transferring genes for a given metabolic route from a donor organism into a heterologous recipient lacking that pathway, whose transcriptional apparatus does not recognize the regulatory signals of the donor DNA. Reports of the stress-induced enhancement of spontaneous mutation rates (Cairns et al. 1988; Hall 1990; Foster 1993) suggest that under starvation conditions, recognition by the host of the transcription signals of the donor DNA can be adjusted to the host transcriptional milieu (Fig. 1). Here we present experimental evidence that when the histidine biosynthetic genes are transferred from a donor bacterium to a heterologous His^- strain initially unable to

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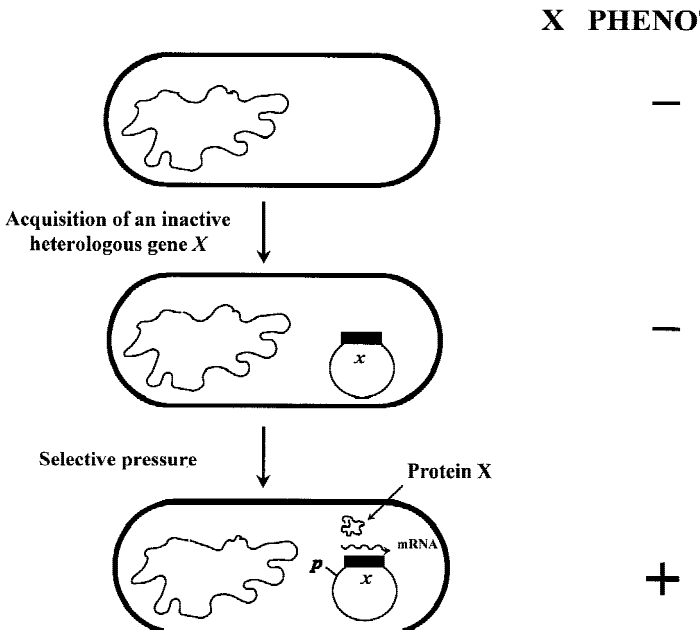

Fig. 1. Model for the expression of newly acquired metabolic genes by promoter-creating mutations in a host cell lacking the function encoded by a gene *X* which has an X^- phenotype. This cell may acquire (by xenology or sinology) a heterologous gene *X* whose regulatory signals are not recognized by the transcriptional apparatus of the host cell; therefore the cell phenotype is still X^-. The appearance of a cell with an X^+ phenotype is possible if, under starvation conditions requiring the function X, mutations falling upstream of the gene *X* create a promoter sequence and render the gene *X* expressable. p, promoter.

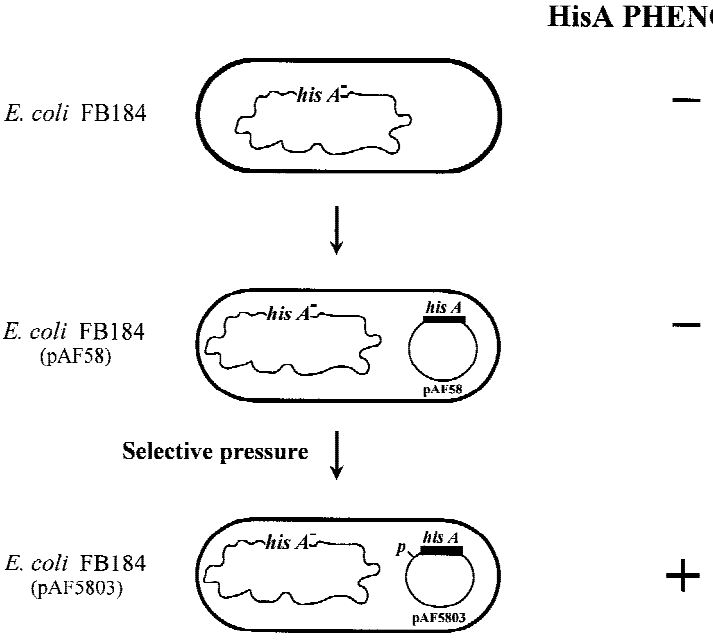

Fig. 2. Overall experimental strategy to detect mutations leading to promoter-like sequences in an *E. coli* $HisA^-$ population under selective conditions, i.e., the absence of histidine in the medium. A similar strategy has been adopted to isolate $HisB^+$ or $HisF^+$ revertants from *E. coli* strains FB251 and FB182 (see text for explanations).

recognize the transcriptional signal of the donor gene(s), regulatory mutations occurring under stress conditions can lead to the activation of the donor DNA by the host RNA polymerase on a very short time scale (Fig. 1).

Methods and Results

Overall Experimental Strategy

The experimental strategy used in this work is based on the utilization of the *Azospirillum brasilense* histidine biosynthetic genes, whose cloning, nucleotide sequence, and transcriptional analysis have been reported elsewhere (Bazzicalupo et al. 1987; Fani et al. 1989, 1993). In this nitrogen-fixing α-proteobacterium seven *his* genes are clustered in an operon of approximately 4.7 kb, which are transcribed in a single polycistronic mRNA. Five of these genes are known to share significant levels of sequence similarity with the corresponding *Escherichia coli his* genes (*hisBd, hisH, hisA, hisF,* and *hisE*) (Fani et al. 1989, 1993, 1995). This operon lies within a 9-kb *Eco*RI DNA fragment unable to complement the *his* mutation of the *E. coli* strains FB251 *hisB855recA56*

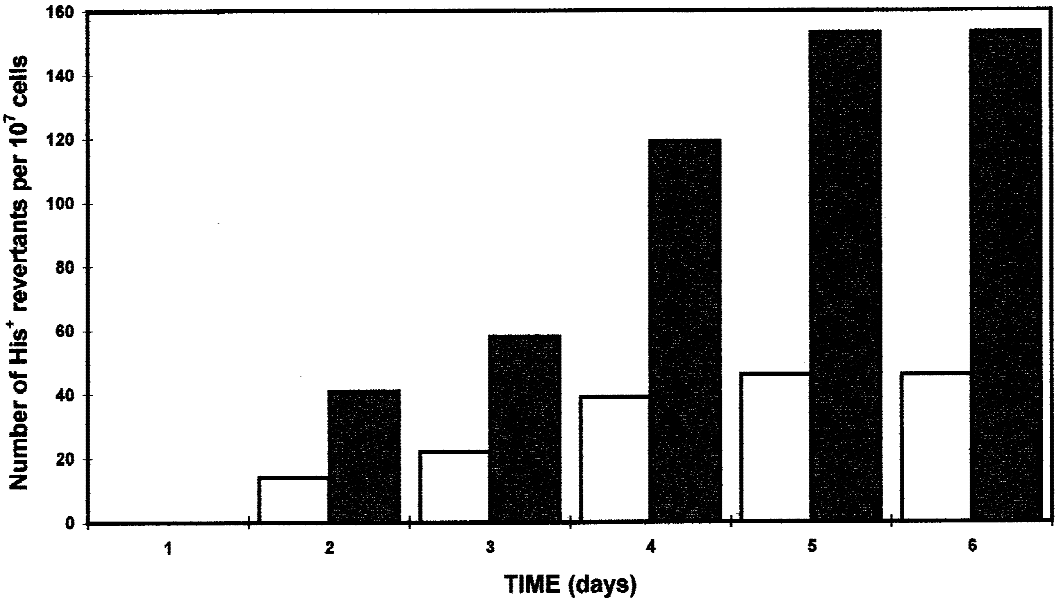

Fig. 3. Accumulation of HisA$^+$ revertants on minimal medium plates without histidine (*white columns*) or in the presence of traces (0.25 μg/ml) (*gray columns*) as a function of time.

(Grisolia et al. 1982), FB184 *hisA915* (Goldschmidt et al. 1970), and FB182 *hisF892* (Goldschmidt et al. 1970) when cloned into the *Eco*RI site of the low-copy number 20-kb plasmid vector pRK290 *tet* (Ditta et al. 1980), resulting in the recombinant plasmid pAF58 (Bazzicalupo et al. 1987). The inability of plasmid pAF58 to complement the *E. coli hisA, hisB,* and *hisF* mutations is due to inefficient recognition of the *A. brasilense his* promoter by the *E. coli* RNA polymerase (Fani et al. 1989). The working hypothesis used here is that under the stress conditions induced by the absence of histidine, the selective pressure would lead to the growth of *E. coli* His$^-$ strains harboring the plasmid pAF58 due to the appearance upstream of the *A. brasilense his* gene cluster of mutations generating promoter-like sequences which are efficiently recognized by the *E. coli* RNA polymerase (Fig. 2).

Table 1. Phenotype of *E. coli* strains FB182 *hisF892,* FB184 *hisA915,* and FB251 *hisB855 recA56* harboring different plasmids

Plasmid	Vector	*his* insert orientation[a]	Phenotype HisA	HisB	HisF
pRK290	pRK290		–	–	–
pAF58	″	f	–	–	–
″	″	r	–	–	–
pAF5803	″	f	+	+	+
″	″	r	+	+	+
pAF5803/1	pLAFR1	f	+	+	+
″	″	r	+	+	+
pAF5803/2	pUC18	f	+	+	+
″	″	r	+	+	+

[a] f, forward; r, reverse.

Isolation of His$^+$ Revertants

The *E. coli* strain FB184 *hisA* (pAF58) was grown overnight at 37°C in Davis minimal medium (MMD) (Davis and Mingioli 1950) containing histidine (25 μg/ml). The culture was then diluted in the same medium at an OD_{550} = 0.1 and incubated at 37°C with shaking. At the end of the log phase, cells were collected and plated in MMD containing tetracycline (10 μg/ml) and in the absence or the presence of histidine (0.25 μg/ml). Appropriate diluitions of cultures were also plated in MMD containing tetracycline (10 μg/ml) and histidine (25 μg/ml) to obtain the number of total viable cells. Plates were then incubated at 37°C and the appearance of His$^+$ colonies was controlled for 8 days. The total number of cells was determined by accurate counts made every day. Results are summarized in Fig. 3 and showed that (a) His$^+$ colonies appeared on selective medium plates after at least 2 days of incubation at 37°C; (b) the number of His$^+$ colonies was always greater in plates containing traces of histidine than in those without histidine; and (c) no His$^+$ revertants were found in the control experiment performed with *E. coli* strain FB184 (pRK290) (data not shown). Similar results (not shown) were observed with strains FB251 (pAF58) and FB182 (pAF58).

Preliminary Characterization of Plasmid DNA from His$^+$ Revertants

The possibility that restoration of prototrophy of His$^+$ revertants could be due to a retromutation in the host

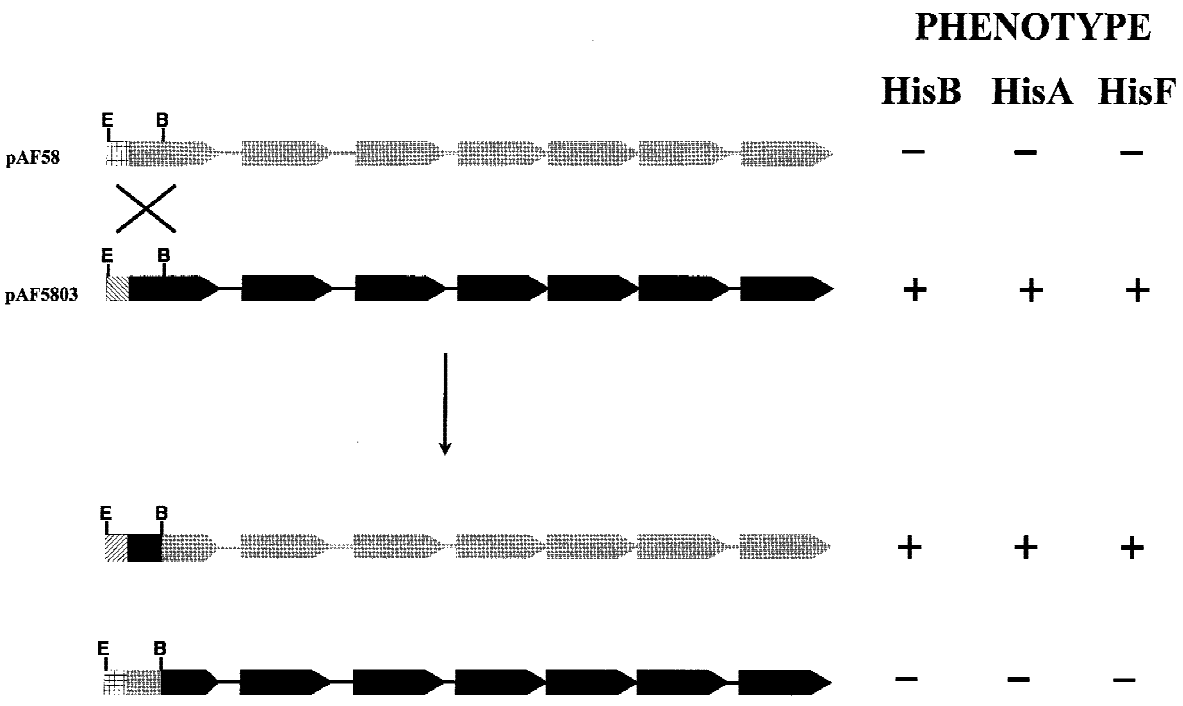

Fig. 4. Effect of the reciprocal exchange of the 469-bp *Eco*RI (E)–*Bam*HI (B) DNA fragment from plasmids pAF58 and pAF5803 on the phenotype of *E. coli* strains FB182, FB184, and FB251.

chromosomal *his* gene or to a recombinational event between the plasmid-borne *A. brasilense his* gene and the *E. coli* chromosomal homologue cannot be excluded a priori, although the different GC contents of the two microorganisms suggest that the second possibility is much less likely. Because of this, plasmid DNA (hereinafter pAF5801, pAF5802, and pAF5803) was extracted from three $HisA^+$ revertants and used to transform competent cells of strain FB184 following the method of Hanahan (1983). All the transformants, selected in LB medium in the presence of tetracycline, were also $HisA^+$, suggesting that the genetic change(s) responsible for the appearance of $HisA^+$ revertants took place within the plasmid molecules, and not in the chromosomal *E. coli hisA* mutated gene. Identical results were obtained with ''mutant'' plasmids extracted from $HisF^+$ and $HisB^+$ revertants (not shown). This putative mutation did not increase the plasmid copy number, as shown from the analysis performed on four ''mutant'' plasmids extracted from His^+ revertant strains FB182(pAF58), FB184(pAF58), and FB251(pAF58), which showed that the copies of these plasmids in a single cell were identical (one or two per cell) to those of plasmid pAF58 (data not shown).

Moreover, restriction analysis with *Eco*RI and *Pst*I of ''mutant'' plasmids extracted from $HisA^+$ revertants of strain FB184(pAF58) showed that their digestion patterns were identical to those of plasmid pAF58, suggesting that no (large) rearrangements took place in ''mutant'' plasmids (results not shown) and that the mutation(s) responsible for the $HisA^+$ phenotype was (were) a point mutation(s). In addition, the fact that the *A. brasilense* 9-kb DNA fragment was able to restore the His^+ phenotype of strain FB184 when it was cloned (in both orientations) onto two other plasmids, the low-copy number cosmid pLAFR1 and the high-copy number plasmid pUC18 (Table 1), suggested that the mutation took place within the *A. brasilense* 9-kb DNA fragment, and not in the vector molecule. Thus, the mutation(s) reported here rendered (at least) the *A. brasilense hisA* gene transcribable and expressable by the *E. coli* transcription apparatus. The available data suggest that this mutation is a regulatory one and is localized upstream of the *A. brasilense hisA* gene. To check this hypothesis, the three ''mutant'' plasmids described above were introduced by transformation into the other two strains, i.e, FB182 *hisF* and FB251 *hisB.* Transformants were then checked to grow in minimal medium in the absence of histidine. As shown in Table 1, all three plasmids were able to restore the His^+ phenotype also in strains FB182 and FB251.

This body of genetic data suggests that (at least) one mutation falling in the *A. brasilense* DNA fragment upstream of the first gene of the *his* operon may have led to a regulatory sequence efficiently recognizable by the *E. coli* RNA polymerase. To verify this possibility, the 450-bp *Eco*RI–*Bam*HI fragment containing the first 100 nucleotides of the *hisBd* gene and its upstream region of plasmid pAF58 was replaced with the corresponding fragments from plasmid pAF5801,

pAF5802, or pAF5803, and vice versa (Fig. 4). The resulting switch prototrophy ⇔ auxotrophy supported this hypothesis.

Discussion and Conclusions

Changes in promoter activity resulting from the introduction of insertion sequences and other types of DNA rearrangements played an important role in the rapid evolution of new functions and control pathways (Cullum and Saedler 1981). Based on the enhancement of spontaneous mutation rates under strongly selective stress conditions (Cairns et al. 1988; Hall 1990; Foster 1993), Kasak et al. (1997) have shown that a combination of base substitutions, deletions, and insertion of the transposon Tn*4652* led to the emergence of promoter-like sequences in a promoterless phenol-utilizing (Phe^+) mutant *Pseudomonas putida* population in the presence of the substrate, but not in its absence. The system described here differs from that of Kasak et al. (1997) in that genetic changes under directed evolution conditions were studied by transferring a *his* gene cluster from the donor *A. brasilense* into a low-copy number plasmid to *E. coli*, whose transcription apparatus is not able efficiently to recognize the donor *his* promoter. As shown here, under stressful starvation conditions the rapid accumulation of regulatory mutations leads to the selection of *E. coli* His^+ colonies which survive in the absence or in the presence of histidine.

Expression of metabolic genes acquired by lateral transfer events can be impaired due to differences in promoter sequences and other control mechanisms, which would result in an inefficient recognition by the transcription system of the host cell. In principle, expression of the newly acquired genes should be more efficient among closely related organisms. Although *E. coli* and *A. brasilense* are closely related proteobacteria (Woese 1991), their phylogenetic separation is reflected in the inefficient recognition of the *A. brasilense his* promoter by the *E. coli* RNA polymerase, which indicates that their corresponding regulatory sequences diverged after their evolutionary separation (Fani et al. 1989). Moreover, the existence of a strong difference between the *his* regulatory signals of these two proteobacteria is supported by the finding that in *A. brasilense* there is an unusual coincidence between the transcription and the translational start points, a feature that *A. brasilense* shares with the Gram-positive soil bacterium *Streptomyces coelicolor* (Fani et al. 1993). As shown here, under laboratory conditions such differences can be overcome in a short time scale by genetic changes that are selected under nutrient-limitation conditions and that may enhance weak promoter activity and/or lead to the mutational adjustment of functional, regulatory sequences.

The results presented here suggest that genetic changes that are advantageous under highly selective, nongrowing conditions may have played a major role in metabolic evolution by allowing newly acquired genes to become fully integrated into the preexisting control systems of the cell. This versatility could have led to the rapid evolution of new functions and regulatory processes in early evolution in which foreign genetic information may have been gained by massive gene transfer and/or cell fusion events. The genetic and molecular characterization of the mutated plasmids is currently in progress and will be reported elsewhere.

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