NEW BIOMEDICAL TECHNOLOGIES

A Method for Modifying the Adhesive Phenotype of Wild Isolates of *Escherichia coli* in Coli-Bacterin Producer Strains

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Use of the suicidal vector pCH103 permitted the creation of recombinant strain with the chromosome DNA site coding for adhesin (FimH) replaced by the kanamycin resistance gene (*npt*). The resultant chimeras transformed by vectors containing *fimH* genes of different origin express type I piles of the relevant phenotype. This method was used to modify *E. coli* M17 producer strain with an unfavorable adhesive phenotype.

Key Words: *coli-bacterin; adhesion; adhesive phenotype*

Coli-bacterin is a probiotic (or eubiotic) representing lyophilized biomass of *E. coli* M17 producer strain. This strain originates from *E. coli* isolated by A. Nissle [11] and serves as the basis for a commercial probiotic Mutaflor (Germany). The commercial preparation Simbioflor (Germany) contains lyophilized biomass of another *E. coli* strain. Strain M17 has been studied in Russia [3]; however, the presence and phenotypical characteristics of type I piles and the adhesive activity related to them were not analyzed.

Recent findings indicate that bacterial cell adhesion to surface receptors is a phenomenon important for understanding the interactions between the microorganism and the host. One route of bacterial adhesion is specific reactions between surface components of the microorganism, which are generally called adhesins, and stereospecific surface receptors. The most prevalent adhesive organelles are type I piles containing D-mannose-sensitive lectin realizing the binding [5]. These fimbria (piles) consist mainly of the structural monomer FimA with slight insertions of accessory subunits FimF, FimG, and the adhesin FimH [6]. Type I piles are characterized by phenotypical and genotypical heterogeneity which is determined solely by the adhesin gene *fimH* [8-10]. Adhesin subclasses differ by the degree of binding to different substrates [10].

In order to investigate the mechanisms of adhesion and its role in the life of bacteria, recombinant variants of wild isolates are needed to alter their adhesive phenotype. In this paper we discuss the preparation of such strains and some other aspects of this problem related to application of this method to the coli-bacterin producer strain.

MATERIALS AND METHODS

Microorganism strains and plasmids. All strains were stored in small aliquots in 15% glycerol at -80°C and grown in LB or BHI (Difco) media at 37°C for 18 h (Table 1).

Manipulations with DNA. Plasmid DNA was isolated on mini-QIAGENE columns (Germany - USA). Restriction, ligation, electrophoresis, and isolation of DNA from gel were performed routinely [1].

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TABLE 1. Strains and Plasmids

	Characteristics
***E. coli* strains**	
PC31[1]	K-12 derivative, gal tonA phx ara
AAEC191A[1]	K-12 derivative, λ-, F-
SM10 (λ*pir*)[1]	thi thr leu tonA lacY supE recA::RP4-2-TC::MuKm λ*pir*
CI10[1]	Isolated in urinary infection
MJ35[1]	- " -
M17[2]	Commercial variant used for producing a probiotic
Mutaflor[2]	- " -
Simbioflor[2]	- " -
Plasmids	
pCH103[1]	pGP704 fimD,G,F, fimH::npt, eda
pPKL9[1]	pBR322 fimB, Ap
pPKL91[1]	pBR322 fimB, Ap, Cm
pGB...[1]	pGB2-24 fimH, Cm
pACYC184[3]	p15A, Cm, Tc
Phages	
p1vir[4]	

Note. [1]From the collection of E. Sokurenko, University of Tennessee, Memphis; [2]Maintained at the Department of Biomedical Technologies, I. M. Sechenov Moscow Medical Academy; [3]NE Biolabs; [4]State Institute of Genetics and Selection of Commercial Microorganisms.

Assessment of adhesive activity. E. coli strains were tested for the capacity to aggregate yeast cells. *Saccharomyces cerevisiae* were resuspended in phosphate buffered saline (PBS, 10 mg/ml). Bacterial cultures were washed in PBS, and suspensions were prepared in 0.1% bovine serum albumin (BSA—PBS) with an optic density of 0.4 at a wavelength of 530 nm, after which they were mixed with yeast suspension. Aggregation was observed visually.

Adhesive activity was assessed by the growth assay [9]. The wells of microtitration plates were coated with substrate (receptors for type I pile adhesin), blocked with BSA—PBS, twice washed in PBS, and incubated for 40 min with bacterial suspension. Unbound cells were washed 6 times with PBS, BHI medium was added into the wells, and the plates were incubated at 37°C during rotation. Light absorbance in each well was measured using an automated microplate reader (Molecular Devices Inc.).

RESULTS

Preparation of recombinant strains of wild isolates of E. coli with defective adhesin gene. The pCH103 plasmid possesses a *pir*-dependent replication system [4,7], due to which it is maintained in the SM10 strain (λ*pir*); this plasmid does not multiply in wild strains. It contains an ampicillin resistance gene, a *mob* domain determining the capacity to mobilization, and a *fim* claster fragment in which the central part of *fimH* gene is replaced by the kanamycin resistance gene (*npt*). Theoretically, during transformation of a microorganism with this plasmid followed by inoculation into selective medium, ampicillin- and kanamycin-resistant colonies appear during one crossing-over. The appearance of colonies resistant only to kanamycin means that a double crossing-over took place, during which the *fimH* gene in the bacterial genome was replaced by the kanamycin resistance gene (Fig. 1).

Calcium transformation is a common method for manipulations with genetic material [1]. However, it turned to be difficult to insert pCH103 plasmid into wild strains. Neither the classical variant, nor the use of modifications of the method with different time of thermal shock, conditions of preparing the competent cells, and other parameters led to isolation of the desired recombinant strains. Attempts to insert plasmid DNA in the cells by electroporation also failed.

The conjugation method [2] was effective with some strains; the selective conditions were the mini-

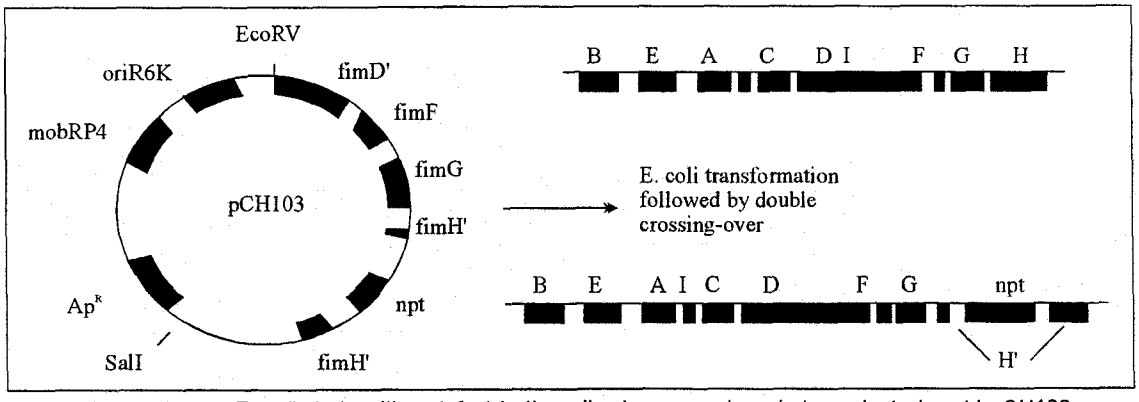

Fig. 1. A scheme of recombinant *E. coli* strain with a defect in the adhesin gene using *pir*-dependent plasmid pCH103.

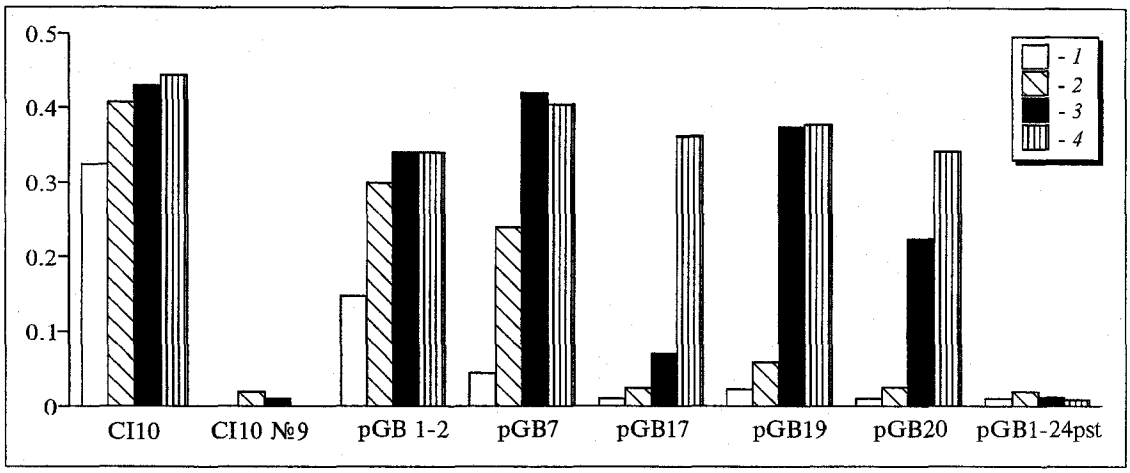

Fig. 2. Adhesive profiles of strain CI10 and its derivative CI10#9 (Δ*fimH*) transformed by pGB plasmids containing different *fimH*. Ordinate: light absorbance growth assay after 3-h incubation. *1*) control (bovine serum albumin); *2*) fibronectin; *3*) mannan; *4*) RNAse B.

mal medium for donor (SM10 does not grow in the minimal medium) and kanamycin resistance for the recipient. The resultant Km^R-colonies were then tested for the sensitivity to ampicillin (Ap). Thus, we obtained ΔfimH variants of CI10, PC31, and MJ35 strains. The frequency of one recombinant event for these strains was 10^{-2}-10^{-3} and of two events 10^{-5}-10^{-6}.

Investigation of adhesive profiles of the resultant recombinant strains. Studies of adhesion confirmed the absence of mannose-sensitive adhesion in the resultant strains. Then these recombinant strains were transformed by plasmids carrying *fimH* genes of different origin, determining different adhesion phenotypes, and containing *fimB* gene which regulates the triggering of type I fimbria synthesis. Adhesiveness of the resultant transformants was investigated (Fig. 2). Obviously, we obtained stable constructions from wild isolates of *E. coli*, in which the adhesive phenotype can be altered by transcomplementation. Such strains can be used for the creation of reliable models in animal experiments for assessing the behavior of microorganisms in a host and for other purposes.

Investigation of adhesiveness of coli-bacterin M17, Simbioflor, and Mutaflor producer strains. The results indicate that M17 and Mutaflor possess a similar highly adhesive M^H-phenotype and Simbioflor — a poorly adhesive M^L-phenotype (Table 2). For growth assay, the cultures are "enriched" for type I piles. We transformed the initial strains by pPKL9 plasmid, containing the *fimB* regulatory protein gene which includes the pile expression. Investigation of the initial Mutaflor strain showed that the level of adhesive activity in the population is very low, in contrast to that of M17 strain. This is probably due to shift of the system regulating the phase variations in type I pile expression toward the "switched off" position. M17 strain could lose this property in the course of manipulations and thus acquire an unfavorable adhesive phenotype, which had to be changed or the system of phase sequence regulation was to be readjusted.

Creation of M17 ΔfimH. It is possible to alter the adhesive phenotype by creating a recombinant strain with deletion at the *fimH* gene region. But the above method failed during experiments with strain M17. We have suggested that a low frequency of transformations prevented the creation of the desired construction, for it did not allow sufficiently large sample for attaining a recombinant event. Therefore, a hybrid plasmid pCHAC2 was created by insertion of pACYC184 vector in pCH103 in SalI restriction site. Along with the *pir*-dependent *ori*, such a hybrid possesses a common origin of replication from pACYC184. In fact, plasmid pCHAC2, isolated directly from strain M17, transformed the same strain with a frequency 3 orders of magnitude higher than plasmids isolated from other strain. SalI restriction of such plasmid resulted in two

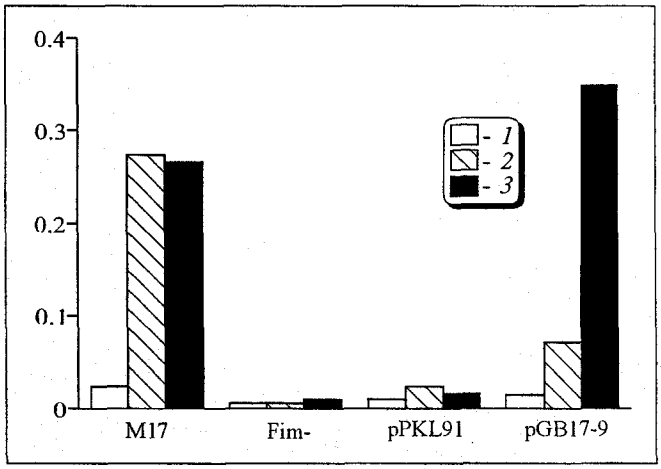

Fig. 3. Growth assay. Ordinate: light absorbance after 2.5-h incubation. M17: intact strain; Fim⁻ — M17Δ*fimH*, pPKL91 and pGB17-9-M17Δ*fimH* transformed by the corresponding plasmids. *1*) bovine serum albumin; *2*) mannan; *3*) RNAse B.

TABLE 2. Results of Growth Assay of *E. coli* Strains M17, Mutaflor, and Simbioflor

Strains tested	Binding to			Phenotype subclass
	RNAse B	mannan	fibronectin	
M17	++++	++++	—	M^H
Mutaflor	++++	++++	—	M^H
Simbioflor	++++	+	—	M^L

fragments, one of which (about 10,000 base pairs), corresponding to plasmid pCH103, was ligated and reinserted in M17 strain by electroporation. Thus, Km^R,Ap^S,CM^S-colony was obtained and denoted as M17Δ*fimH*.

Investigation of adhesive activity of M17ΔfimH strain. The resultant strain does not contain plasmid DNA. Standard investigations of its adhesive activity, as was done with the Fim^--strains CI10, MJ35, and PC31, showed that its properties were similar. Both intact and transformation-enriched pPKL91 culture showed zero activity of type I piles. Transformation of plasmid pGB17-9 led to a transcomplementation effect, rendering the M^L strain the phenotype determined by the *E. coli* gene F18 contained in the *fimH* plasmid (Fig. 3).

Thus, a *E. coli* strain was obtained, in which recombination resulted in replacement of the initial *fimH* gene for cassette with the kanamycin resistance gene. The method for obtaining such strains ensures minimal changes in genetic system of the cell: the site of changed gene is accurately localized, and the promoters of adjacent genes are aimed towards it, therefore the functions of these structures are preserved. Applied problems are also solved during this operation: the resultant strains can be used in studies of adhesion and its role in stabilization of bacteria as components of normal human microflora or an infectious agent; commercial M17 strain used for producing coli-bacterin not just loses its adhesive phenotype, but becomes capable of substituting it for virtually any desired type of adhesive activity.

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