# Genetic Variability of Enterobacterial Resistant Integrons

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Sulfamide-resistant ($Sul^R$) natural strains of enterobacteria (11.9% of a total of 797 cultures, isolated with enteric microflora of honeybee gut, bee-fermented pollen, and plant issues) were tested for class 1 antibiotic resistance integrons (MRI). Only 5.3% of $Sul^R$ strains were MRI-positive. Mutability of weak (wild type) MRI promotor has been shown. A more active hybrid promotor type has been amplified in isolated *Klebsiella oxytoca* strains. Regulatory genetic modifications in MRI are fraught with the development of multiple drug resistance of opportunistic strains.

**Key Words:** *Enterobacteriaceae; integron; promotor; regulatory mutations; sulfamides*

Integrons as vectors for cloning and expression of adaptive response genes are present in about 10% of completely sequenced bacterial genomes [8]. Hospital infection agents are characterized by the presence of class I resistance integrons (MRI) including a variable set of gene cassettes encoding drug resistance determinants [2]. Sulfamide resistance gene *sul1*, located downstream from the cassettes, is found in more than 90% MRI [9]. The level of gene cassettes expression (phenotypically the minimum inhibitory concentration of antibacterial drug) is determined, except for rare exclusions, by activity of the only promoter located upstream from the gene cassettes. "Weak" and "strong" integron promoters are known; there also transitional uncommon hybrid variants resulting from a series of mutations [10].

*Enterobacteriaceaceae* family bacteria, which have the majority of the known MRI, are a convenient model for studies of natural selection of drug resistant strains [9,13]. Up to 50% enterobacterial strains isolated at medical institutions can be integron-positive [15]. The prevalence of MRI can be evaluated by the PCR amplification of nucleotide sequences of the promotor region [2,7]. However, the degree of the promoter variability remains unclear, particularly in rare opportunistic species. Presumably, new agents of human infections evolve among them, including agents developing under natural conditions [11].

We evaluated the incidence of MRI in natural populations of *Enterobacteriaceaceae* and the dominant type and genetic variability of the promoters in comparison with the variants present in clinical strains.

## MATERIALS AND METHODS

Honey bee *Apis mellifera mellifera L.* in its natural habitat was selected as the bacterial carrier. Due to specific ecology and behavior, the bees "collect" bacterial material at a waist territory and exchange it inside a local population. We studied a total of 797 isolated strains, referred to *Enterobacteriaceaceae* family; the methods for isolation and identification of the cultures have been described in detail previously [1]. The enterobacteria were cultured in Mueller–Hinton's agar; 20% solution of sulfacetamide (625 μl/ml medium) was added for selection. In order to isolate genomic DNA, biomass of individual colonies, grown over 24 h in the presence of sulfacetamide, was subjected to thermal lysis in 100 μl pharmacopoeial water (sterile eppendorf tubes were placed for 15 min into a Termit hard-body thermostat at 95°C), after which the samples were centrifuged (30 sec, 12,500 rpm) for separation of cell fragments.

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Primers for PCR were tested in a previous study [7], their sequences corresponding to nucleotide positions 324-342 and 630-650 according to GeneBank (acc. no. AB469046). Reagents for PCR were used according to recommendations of polymerase manufacturer (SibEnzym) with 2 U Taq DNA-polymerase per 50 μl reaction mixture, but with 0.5 mM deoxynucleoside triphosphate solution. The reaction was carried out in a MyCycler apparatus (BioRad) according to our protocol ruling out nonspecific amplification at the primers annealing temperature of 60°C and with DNA elongation time at least 90 sec. The size of the resultant amplicons (327 n. p.) was confirmed by agarose gel electrophoresis with DNA marker 100-1000 n. p. (SibEnzym). The PCR products were collected on gel plates in an Invitrogen device and subjected to two-way automated sequencing in a MegaBACE 1000 capillary system (GE Healthcare Life Sciences) using a DYEnamic ET Dye Terminator Cycle Sequencing Kit. *Acinetobacter* (strain 21) DNA served as positive control for PCR [3], phage T7 DNA as negative control.

## RESULTS

In virtually all tested samples, 8-14% isolated strains (a total of 11.9%) maintained their growth in the presence of soluble sulfamide, which meets the criteria of selecting MRI with actively expressed *sul1* dihydropteroate synthetase gene (Table 1). However, the promotor region of MRI and part of DNA sequence essential for gene cassette recombination were amplified in PCR in just 5 of 95 (5.3%) sulfamide-resistant cultures (the fragments are presented in Fig. 1). Hence, the study confirmed very low incidence of MRI in bacteria under natural conditions without verified antibacterial selection. By contrast, the number of isolated strains resistant to sulfamide drugs proved to be unexpectedly high, *e.g.* for the *Pantoea agglomerans* plant pathogen; several strains of this pathogen were described among infection agents in a pediatric clinical hospital [6]. Integron-positive strains among drug-resistant *P. agglomerans* strains have been described [4], but we detected no MRI in endorphyte or pollen-contaminating strains in our study.

The only integron-positive isolate (laboratory No. 325) referred to *Enterobacteriaceaceae* family by common morphobiochemical methods of identification was found in bee enteric microflora. The primary DNA structure of the promotor region corresponded (by 100%) to the consensus sequence of the "weak" promotor typical of natural integrons, *e.g.* In0, a possible evolutionary ancestor of all MRI [5] (Fig. 1, *a*). However, a mutation in the last triplet of a spacer site between -35 and -10 hexamers was found in the structure of *Klebsiella oxytoca* strain "weak" MRI promotor (Fig. 1, *b*). Nucleotide substitution (C—G) can be a cause of enhanced expression of the gene controlled by the new promotor [10].

The most important result was the detection of hybrid promotor in three *K. oxytoca* strains, isolated from specimens of the bee pollen basket (Fig. 1, *c*). Discussing the MRI variability phenomenon, we should remember that the "stem-loop" structures in the recombination site of the gene cassette are transcription terminators and hence, the expression of many cassettes by the "weak" promotor is difficult, and only modification towards the hybrid type can partially reduce this limitation. The number of transcrips detected for the gene cassette increases two-fold in the pre-

**TABLE 1.** Basic Characteristics of Isolated *Enterobacteriaceaceae* Strains

| Source of isolates | Total number of isolates/number of $Sul^R$ isolates | Number of integron-positive isolates (species) |
|---|---|---|
| Enterobacteria isolated from the gut of three bee families in winter-spring | 222/29 | 1 (species not identified) |
| Enterobacteria isolated from the gut of bees collected in the Perm territory | 202/17 | 0 |
| *Pantoea agglomerans* representatives isolated from specimens of bee pollen basket | 153/21 | 0 |
| *Pantoea agglomerans* representatives isolated from the surface and internal tissues of herbal plants | 25/6 | 0 |
| Enterobacteria isolated from specimens of bee pollen basket in sanitary bacteriological studies | 195/22 | 4 (4 (all *Klebsiella oxytoca*) |
| Total | 797/95 | 5 |

**Note.** $Sul^R$: isolated strains growing in sulfacetamide-containing Mueller–Hinton medium.

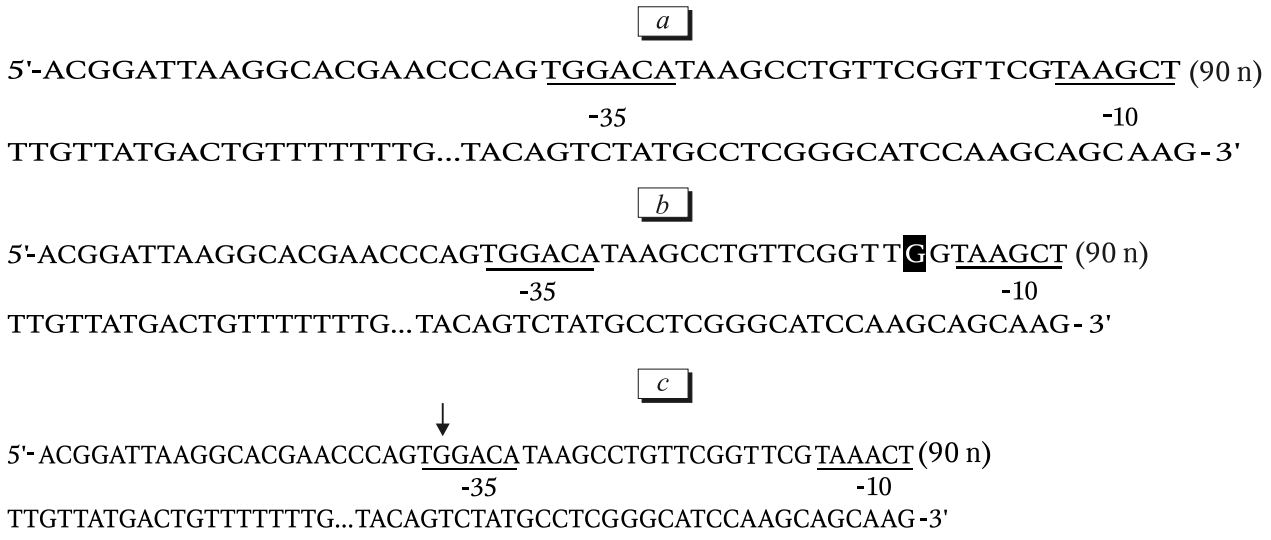


**Fig. 1.** Nucleotide sequences of weak (*a*, *b*) and hybrid (*c*) MRI promoters: hexamers -35 and -10 are underlined; mutation in the spacer sequence is shown against the black background. Dotted line shows positions absent in the presented sequences, but essential for stimulation of expression activity in clinical strains [12]. Substitution of guanine shown by arrow leads to modification of the hybrid promotor into a strong one.

sence of a modified variant, while the minimum inhibitory concentration of the antibiotic can increase up to 4-fold [13]. A single substitution of guanine (G) for thymine (T) in position 2 of -35 hexamer is sufficient for further modification of the hybrid promotor with a 10-fold increase of activity [12]. Numerous agents of hospital infections have been described among *Klebsiella* genus representatives [14]. The majority of these strains are characterized by acquired multiple drug resistance, including that introduced by integrons. However, we failed to detect other genetic variants of clinical strains, associated with an additional increase of the integron promotor activity, for any of the amplified sequences (Fig. 1).

Hence, genetic organization of MRI promoters even in natural enterobacterial strains attests to the presence of the mutation process increasing their activity. The prevalence of these MRI among bacterial strains and species can be maintained by association with plasmids of an extensive range of hosts and transposons and enables the host bacteria resist the antibacterial chemotherapeutic drugs [15].

It is noteworthy that there are no barriers precluding infection of inpatients and outpatients (for example, suffering from diseases of the upper airways) by MRI strains disseminated with plant pollen. The need in further studies is explained by the fact that pollen of plants pollinated by insects (in our study bee enzyme-treated pollen basket) differs from the pollen in the wind-pollinated plants [3].

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