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Review

Gene-cassette for adaptation of *Lactococcus lactis* to a plant environment

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Abstract – The generally accepted opinion is that the natural niche for lactococci are plants. Several genes reminiscent of the environmental adaptation of these bacteria to the plant habitat were found as a result of our work on the pullulanase coding region in the *Lactococcus lactis* IBB500 strain. All genes were located within an 11-kb DNA fragment of a 35-kb plasmid. Analysis of the nucleotide sequence of the 11-kb DNA fragment showed three regions: (i) a middle region – encoding the potential pullulanase operon (ii); a pullulanase upstream region – encoding two cold shock proteins; and (iii) a pullulanase downstream region – encoding two cadmium resistance proteins. Analysis of the phylogenetic relationships of these regions through inspection of their G+C content and codon preference, as well as homology of their protein products and constructions of phylogenetic trees strongly supported the hypothesis of their acquisition through a horizontal gene transfer. Focusing further research on the middle region we have found that the pullulanase gene, belonging to the putative *pul* operon, was preceded by a long, non-translated region in which five putative promoter sequences were identified. Moreover, a DNA sequence similar to the highly conserved *cre* box involved in glucose repression was found in front of one of them. The hypothesis of glucose repression was further tested using transcriptional fusion between the *pul* and *luxAB*-reporter genes, located on the parental plasmid in the *L. lactis* IBB500 strain.

***Lactococcus lactis* / adaptation / gene-cassette / horizontal gene transfer / pullulanase**

Résumé – Une cassette de gènes pour l'adaptation de *Lactococcus lactis* à l'environnement végétal. Une opinion largement acceptée est que les plantes constituent une niche naturelle pour les lactocoques. Nos recherches ont montré que plusieurs gènes impliqués dans une possible adaptation environnementale de ces bactéries aux plantes sont localisés dans la région codant une pullulanase chez la souche *Lactococcus lactis* IBB500. Tous ces gènes se trouvent sur un fragment d'ADN d'une taille de 11 kb, situé sur un plasmide de 35 kb. L'analyse de la séquence nucléotidique de ce fragment de 11 kb a révélé 3 régions : (i) une région centrale – avec la présence d'un opéron potentiel de pullulanase, (ii) une région en amont – codant 2 protéines du choc au froid et (iii) une région en aval – codant 2 protéines de la résistance au cadmium. L'analyse phylogénique de ces régions suggère que ces gènes ont été acquis par un mécanisme de transfert horizontal. L'étude de la région centrale a montré que le gène de la pullulanase est précédé par une longue région non codante, dans laquelle nous avons identifié 5 promoteurs potentiels. De plus, une séquence nucléotidique similaire à la boîte *cre*, très conservée et impliquée dans la répression par le glucose,

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a été trouvée en amont d'un de ces promoteurs. Nous avons testé cette hypothèse de répression par le glucose en utilisant la fusion transcriptionnelle entre le gène *pul* et les gènes rapporteurs *luxAB*, placée sur le plasmide parental chez la souche *L. lactis* IBB500.

***Lactococcus lactis* / adaptation / cassette / transfert horizontal de gènes / pullulanase**

1. INTRODUCTION

It is believed that a plant environment constitutes the natural niche for lactococci. This implies that these bacteria should possess a metabolic potential which helps them to survive and to live in this habitat. One could expect then, that lactococci might degrade plant sugars in order to use them as energy and carbon sources. In the plant world, the most abundant sugars are cellulose, sucrose and starch.

Despite the lack of cellulose degrading enzymes in lactococci these bacteria were shown to produce those, splitting the other two sugars – disaccharide sucrose, and most recently, a polysaccharide starch [8]. Moreover, lactococci are capable of assimilating β-glucosides, such as cellobiose, salicin, arbutin and esculin, which also belong to plant sugars [2]. Interestingly, it seems that some of the β-glucosides, e.g. cellobiose, can modulate the environmental adaptation of these bacteria and prompt them to grow in milk, another habitat of lactococci. It has been demonstrated that low concentrations of cellobiose induce a β-glycosidase (or P-β-glycosidase) activity in plasmid-free, lactose-negative *Lactococcus lactis* IL1403 cells, that enables them to hydrolyze lactose, the main carbohydrate present in milk [1]. These observations suggest that there is a kind of coupling between cellobiose and lactose assimilations in *Lactococcus lactis*.

It has been demonstrated that amylolytic activity of the *L. lactis* IBB500 strain, isolated from a cow's milk sample collected at a farm, is encoded by a gene located on a 35-kb plasmid [8]. These experiments showed that various sugars modulate production of the enzyme secreted to the medium; starch was found to be the best inducer, while glucose strongly repressed amylolytic activity.

Here we discuss the question of whether the plasmid gene, coding for the amylolytic enzyme, is a sole element of adaptation of the *L. lactis* IBB500 strain to a plant environment.

2. GENETIC ORGANIZATION OF THE PULLULANASE CODING REGION

Detection of the amylolytic activity and identification of the producer strain relied on a simple plate test [8]. In this assay, on starch-supplemented medium, a strain producing an amylase was surrounded by a halo uncolored by the Lugol solution, as a result of starch degradation. Among the 100 lactococcal strains tested, one, *L. lactis* IBB500, was found to produce an amylase.

Once the gene coding for the amylolytic enzyme was found to be localized on the 35-kb plasmid in the *L. lactis* IBB500 producer strain, it was subsequently cloned and sequenced (Doman-Pytka, manuscript in preparation). These data demonstrated that the amylolytic enzyme is highly homologous to bacterial pullulanases, and therefore its corresponding gene was called *pulA* [3; accession number AX74163]. DNA sequencing of the vicinity of the *pulA* gene revealed a 11-kb DNA contig in which 3 regions can be distinguished: (i) a middle region – encoding the putative pullulanase operon, containing *pulA* and 3 other *orfs*; (ii) a pullulanase upstream region; and (iii) a pullulanase downstream region (Fig. 1).

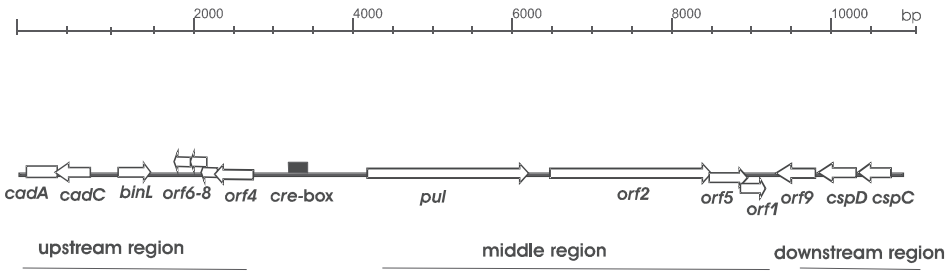

Figure 1. Schematic organizations of *orfs* within three regions of the 11-kb contig of the pIBB500 plasmid from the *L. lactis* IBB500 strain. Only those *orfs* whose protein translation products display homology to known proteins are named.

Table I. Identification of homologues of some of the *orfs* present in the vicinity of the pullulanase coding sequence of the pIBB500 plasmid of the *L. lactis* IBB500 strain.

Gene	Protein	Bacterium	Amino acid identity (%)	Amino acid similarity (%)
cadA	Putative cadmium efflux ATPase	*Listeria monocytogenes*	63	80
cadC	Cadmium resistance regulatory protein	*Listeria innocua*	100	100
binL	Resolvase of Tn552	*Staphylococcus aureus*	78	90
cspD	Cold shock protein CspD	*Lactococcus lactis*	91	97
cspC	Cold shock protein CspC	*Lactococcus lactis*	96	98

2.1. Gene content of the pullulanase upstream region

Seven *orfs* were identified in the pullulanase upstream region (Fig. 1). All of these *orfs*, except one, were divergently oriented in respect to the transcriptional orientation of the pullulanase putative operon. Of these 7 *orfs*, 2 displayed amino acid homology to genes conferring resistance to cadmium in *Listeria*, and were therefore called *cadC* and *cadA* (Tab. I). This pair of genes codes for a regulatory protein and a putative ATPase involved in cadmium transport out of a cell, respectively. Both lactococcal proteins displayed a high level of amino acid identity, more than 60%, to their listerial protein counterparts. However, while the putative cadmium resistance regulatory protein from *L. lactis* IBB500 was similar to its counterpart from *Listeria innocua*, the putative lactococcal ATPase resembled its homologue from *Listeria monocytogenes*. It should also be mentioned that in the chromosome of the model strain *L. lactis* ssp. *lactis* IL1403 a *cadA* gene, coding for a cadmium efflux ATPase, is present, while there is no *cadC* gene [4]. Moreover, the homology level between the lactococcal plasmidic and chromosomal copies of the *cadA* is less important than that between lactococcal plasmidic and listerial copies.

The other 4 *orfs* present in this pullulanase upstream region shared homology with lactococcal genes coding for unknown proteins.

2.2. Gene content of the pullulanase downstream region

Downstream of the *pulA* region a set of 3 *orfs* was identified (Fig. 1). Their transcriptional orientation was convergent to genes present in the pullulanase middle region. The most extreme pair of the *orfs*

coded for protein products highly homologous to the cold shock proteins from *L. lactis*, and were therefore called *cspC* and *cspD* (Tab. I). Both the G+C content, found to be 35% in *cspC* and 33% in *cspD*, as well as the codon preference of these two *orfs* were similar to lactococcal genes. While *cspD* seems to be another copy of that present in the lactococcal chromosome [4], the *cspC* could be a unique one and restricted to the plasmid DNA in the *L. lactis* IBB500 strain. What the advantage of this second copy of *cspD* is for the bacterial cell, remains to be elucidated.

3. ADAPTATION GENE-CASSETTE

Lactococci inhabit two main environments – milk and plants. Many genes important for the propagation of these bacteria in milk have been found on various mobile elements, such as plasmids or transposons. Less data are available concerning the adaptation of *L. lactis* to the plant ecosystem. One could argue that lactococci implemented into a plant habitat should possess a metabolic potential that enables them to manage with several different, non-optimal or even negative factors, in order to survive and persist in this niche.

We have found that the 11-kb DNA fragment of the 35-kb plasmid present in the wild-type *L. lactis* IBB500 strain fulfils such a demand of bacterial cells. At first, genes present on this 11-kb fragment help lactococci to use starch, an abundant plant storage carbohydrate, as a carbon and energy source. Secondly, a pair of genes, coding for cold shock proteins, represents an advantage for these bacteria to persist at low temperatures. Thirdly, another pair of genes, *cadC* and *cadD*, helps lactococci to survive in a chemically-polluted plant ecosystem. It is worth noting that bacteria resistant to heavy metals such as cadmium can be isolated from various environments, e.g. soil or water, which are contaminated with these metals.

An interesting point to study is whether the *L. lactis* IBB500 strain acquired cadmium resistance genes as a result of its adaptation to a cadmium-contaminated environment or as a result of co-culturing with a *Listeria* strain, which contaminated milk or fermented milk products, and therefore persisted together in the same environment. To discriminate between these hypotheses is not easy, since the GC% as well as patterns of codon preference in *Lactococcus* and *Listeria* are very similar. Analysis of the G+C content of the *cadC* and *cadA* homologues in the *L. lactis* IBB500 strain showed values of 32% and 33%, respectively, which are close to the mean value 36% G+C for both of the bacterial genera.

We propose that the 11-kb plasmid DNA fragment represents a gene-cassette, or is a part of a larger one, that is important for lactococci for their adaptation to the plant environment. It seems probable that at least some of these genes were acquired through a horizontal gene transfer. There have already been several examples of lateral gene transfer between microbial genomes [6, 9, 10] including lactic acid bacteria [4, 7, 11]. The important role of plasmids and phages in bacterial evolution and environmental adaptation is also emphasized [12].

4. GLUCOSE REPRESSION OF THE PULLULANASE GENE

Focusing further research on the middle region we found that the pullulanase gene, belonging to the *pul* operon, was preceded by a long, non-translated region in which five putative promoter sequences were identified. Moreover, a DNA sequence similar to the highly conserved *cre* box involved in glucose repression was found in front of one of them (Doman-Pytka et al., manuscript in preparation). The hypothesis of glucose repression was further tested using transcriptional fusion between the *pul* and *luxAB*-reporter genes [5, 13], located on the parental plasmid in the *L. lactis* IBB500 strain (Tab. II). These experiments showed that the *pul* promoter activity in the presence of starch was 10^5 times higher than in

Table II. The effect of glucose on the expression of the pullulanase gene from the *pul:lux*AB transcriptional fusion in *L. lactis* IBB500.

Luciferase activity (Lux/OD) in M17 cultures containing sugars		
2% glucose	1% galactose	1% starch
5	10^4	2.5×10^5

the presence of glucose. This suggests that the expression of the *pulA* gene is controlled by a regulatory mechanism of glucose repression.

5. CONCLUSION

Analysis of the nucleotide sequence of the 11-kb DNA fragment from the pIBB500 plasmid of the *L. lactis* IBB500 strain revealed the presence of several genes reminiscent of lactococcal adaptation to their natural habitat – the plant ecosystem. Therefore, we propose that the 11-kb plasmid DNA fragment represents a gene-cassette, or is a part of a larger one, that is important for lactococci for their adaptation to the plant environment.

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