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Analysis of Exopolysaccharide Production by *Lactobacillus casei* CG11, Isolated from Cheese

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Exopolysaccharide-producing *Lactobacillus casei* CG11 was isolated from soft, white, homemade cheese. In basal minimal medium, it produces a neutral heteropolysaccharide consisting predominantly of glucose (about 75%) and rhamnose (about 15%). Plasmid curing experiments revealed that exopolysaccharide production by strain CG11 is linked to a plasmid approximately 30 kb in size.

Different bacterial species produce exopolysaccharides (EPSs), located outside the cell wall, that result in mucoid, slimy colonies (19). It is known that microbial production of EPS and sugar composition are both influenced by culture conditions. In addition, EPS production is stimulated by excess carbohydrate in the growing medium and by low temperatures (2, 16, 17, 18). The ropiness of thermophilic and mesophilic lactic acid bacteria is related to EPS production (for a review, see reference 5). One lactic acid bacterium, *Lactococcus cremoris*, was isolated from Swedish sour milk and Finnish fermented milk (11). Slime-producing strains of *Lactobacillus hilgardii* and a *Leuconostoc* sp. were isolated from kefir grains (14).

When it grows in skim milk, *Streptococcus salivarius* subsp. *thermophilus* produces a heteropolysaccharide that is predominantly composed of galactose and glucose (7). The growth of *Lactobacillus bulgaricus* on skim milk medium results in production of heteropolysaccharide that contains galactose, glucose, and rhamnose (6). Recently, homo- and heterofermentative *Lactobacillus* species were isolated from sugary kefir grains. One heterofermentative species, related to *L. hilgardii* NCDO264, produced polysaccharide from sucrose when grown in MRS sucrose broth (15). In this report we describe an EPS-producing strain, *Lactobacillus* sp. strain CG11, which was isolated from soft, white, homemade cheese. The G+C content (43.4 mol%) and the pattern of carbohydrate fermentation strongly indicated that the isolate is *Lactobacillus casei*.

The influence of carbon sources on EPS production by *L. casei* CG11 was analyzed by its growth in MRS broth (Difco, Detroit, Mich.) containing 2% sugars. An overnight culture (16 h) of strain CG11 in MRS broth containing glucose was inoculated (0.1%) into a set of MRS broth cultures supplemented with various sugars and incubated for 24 h at 25°C. EPS appeared to be produced in all MRS cultures containing sugars that were fermentable by the strain CG11, with the exception of the culture containing fructose. Comparison of culture viscosities revealed that EPS production was the most intensive in the presence of sucrose. Recently, a *Lactobacillus* sp. in which production of EPS was markedly enhanced by the presence of lactose, sucrose, and glucose in the growing medium but not by the presence of fructose or galactose was described (21).

To measure the EPS production and determine the sugar composition of the EPS produced, strain CG11 was grown in basal minimal medium (BMM) (12), a chemically defined medium. This medium allows a more precise measuring of EPS production and monomer determination because it does not contain any of the polysaccharides present in MRS. A preculture was inoculated (0.1%) in BMM containing 1 or 2% glucose or a mixture of 1% glucose and 1% sucrose and incubated at 25°C for up to 72 h. EPSs were isolated from the culture medium by repeated ethanol precipitation (6, 7).

In BMM supplemented with 1 or 2% glucose or a sugar mixture (1% glucose and 1% sucrose), strain CG11 produced 140, 185, and 165 mg of EPS per liter, respectively, after 72 h of growth at 25°C. Comparable amounts of EPS were produced when strain CG11 was grown in rather complex media, such as skim milk or milk ultrafiltrate (8). Generally, independent of the medium in which strain CG11 grew, the yield of EPS was smaller in cultures grown at 30°C than in those grown at 25°C, and the amounts of EPS produced were about 50% lower. This is in agreement with the mechanism proposed by Sutherland (18), who postulated that if cells are growing slowly, then wall polymer synthesis will also be slow, thereby making more isoprenoid phosphate available for exopolymer synthesis. It also seemed that EPS synthesis by strain CG11 continued beyond the stationary phase of growth. In fact, EPS quantities recovered after 72 h of incubation were 30 to 50% higher than those after 48 h of incubation.

The monomer composition of EPS produced in BMM by *L. casei* CG11 was analyzed. Total sugars were determined by the phenol-sulfuric acid method (9) with glucose as a standard; individual sugars were converted to their alditol acetates and analyzed as described previously (3). This analysis revealed that the EPS produced by strain CG11 is a neutral heteropolysaccharide. In addition, the sugar composition the EPS depended on the complexity of the medium in which this strain grew. EPS produced in BMM supplemented with glucose or a mixture of glucose and sucrose was mainly composed of glucose (76.8 or 76.9%, respectively). A relatively high percentage of rhamnose was present in EPS produced under these conditions (13.4 or 18.9%, respectively). In addition to glucose and rhamnose, a very small percentage of galactose was present in EPS and traces of

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TABLE 1. Monosaccharide composition of EPS produced by *L. casei* CG11 grown in BMM[a]

Monomer	% of indicated monomer in EPS from cells grown in:	
	BMM–2% Glc	BMM–1% Glc–1% Suc
Rhamnose	13.4	18.9
Arabinose	0.2	0.2
Mannose	0.5	0.0
Galactose	4.5	1.8
Glucose	76.8	74.5

[a] Glc, glucose; Suc, sucrose. The sugar composition of EPS, given as a percentage of total sugars found, was determined in cultures after 72 h of incubation at 25°C. The values are the means of two determinations.

arabinose were also identified (Table 1). In contrast, the proportion of galactose (55.3%) was higher than that of glucose (39.1%) and rhamnose was completely absent from EPS produced by strain CG11 grown in skim milk (8). It was recently reported that *Klebsiella* sp. strain K32 and *Acinetobacter calcoaceticus* BD4 both produced EPSs with high rhamnose contents (10, 20). However, the rhamnose content of EPS was dependent on the carbon source in the growth medium (4).

EPS production by lactic acid bacteria is unstable. This may be because plasmid DNA is involved in the expression of mucoid (Muc^+) phenotype. Plasmid-encoded Muc^+ phenotypes were found in *L. cremoris* used for the production of Swedish sour milk ("longfil") and Finnish milk ("viili") (13, 22). Transfer of plasmid DNA encoding a Muc^+ phenotype into plasmid-free *Lactococcus lactis* subsp. *lactis* MG1614 was also successful (24). Recently, a correlation between the Muc^+ phenotype and the 4.5-MDa (about 7-kb) plasmid in *L. casei* subsp. *casei* NCIB4114 was found (23).

It has been established that natural isolate *L. casei* CG11 has plasmids. Bearing this in mind, it would be of interest to determine whether there is a relationship between the presence of plasmids in strain CG11 and the Muc^+ phenotype. Therefore, plasmid curing experiments were performed. MRS broth (10 ml) containing either 10 μg of novobiocin per ml, 30 μg of acriflavin per ml, or 50 μg of ethidium bromide per ml was inoculated with a sample (0.1 ml) of overnight culture and incubated at 30°C for 18 h. MRS broth cultures were passaged twice in each curing experiment, and then appropriate dilutions were plated onto MRS agar plates and incubated at 30°C for 48 h. Colonies were picked with toothpicks; those that did not produce long ropy strands were tested for the EPS-producing ability, and their plasmid profiles were analyzed. Plasmid DNA was isolated as described by Anderson and McKay (1). Plasmid curing experiments resulted in the selection of nonmucoid (Muc^-) colonies obtained only after treatment with novobiocin (16% of colonies, CG11-NB derivatives) and sublethal temperature (19% of colonies, CG11-ST derivatives). These Muc^- colonies did not regain the ability to produce EPS during 10 subsequent recultivations at the optimal growing temperature (30°C) in the MRS broth lacking the curing agent. It is worth mentioning that the loss of EPS-producing ability coincided with the inability to ferment α-methyl-glucoside, which is a normal trait of *L. casei* CG11.

Taken together, these data strongly suggest that synthesis of EPS by *L. casei* CG11 is plasmid linked. Indeed, analysis of the plasmid profiles of Muc^- derivatives belonging to either the CG11-NB or the CG11-ST class confirmed that EPS production ability was plasmid linked (Fig. 1). Plasmid

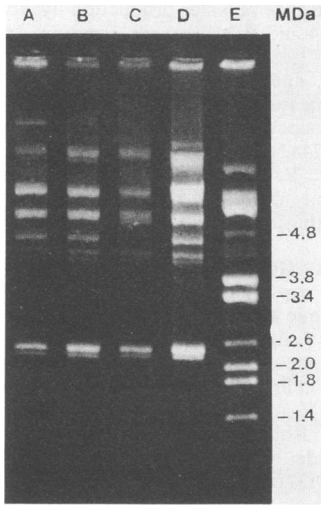

FIG. 1. Plasmid profiles of *L. casei* CG11 (A) and nonmucoid (Muc^-) derivatives CG11-NB (B), CG11-ST1 (C), and CG11-ST2 (D). The plasmids of *Escherichia coli* strain V517 (E) were used as size standards.

curing of the strain CG11 by novobiocin resulted in loss of a plasmid of about 30 kb in CG11-NB derivatives (Fig. 1, lane B). Muc^- CG11-ST derivatives showed two kinds of plasmid profiles. One Muc^- derivative, CG11-ST1, had lost two plasmids (about 30 and 7 kb) (Fig. 1, lane C), whereas the derivative CG11-ST2 was cured of only one plasmid of about 30 kb (Fig. 1, lane D). Therefore, it can be concluded that a plasmid of about 30 kb contains a gene(s) that is involved in some process of EPS biosynthesis or at least in the regulation of EPS production.

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