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Heterologous Expression of the Pneumococcal Serotype 14 Polysaccharide in *Lactococcus lactis* Requires Lactococcal *epsABC* Regulatory Genes▽†

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The pneumococcal serotype 14 polysaccharide was produced in *Lactococcus lactis* by coexpressing pneumococcal polysaccharide type 14-specific genes (*cpsFGHIJKL*$_{14}$) with the lactococcal regulatory and priming glucosyltransferase-encoding genes specific for B40 polysaccharide (*epsABCD*$_{B40}$). The polysaccharide produced by *Lactococcus* was secreted in the medium, simplifying downstream processing and polysaccharide isolation from culture broth.

Capsular polysaccharides (CPSs), either as purified polysaccharides or as protein conjugates thereof (19), have been applied as antigens in several vaccines that are currently marketed. Pneumococci are a common cause of respiratory tract infections (pneumonia) and ear infections (otitis media) but can also cause more life-threatening diseases like meningitis and sepsis (12, 13). Heterologous expression of pneumococcal CPS in nonpathogenic *Lactococcus lactis* has significant advantages relative to the current pneumococcal production process, including the reduced biological containment requirements for *L. lactis.* In addition, a *L. lactis*-based production process is relatively easy to scale up to industrial volumes by using low-cost cultivation conditions based on anaerobic growth and relatively simple medium (Pharma Grade) (9). A third advantage of an *L. lactis*-based production system could be that the polysaccharide is devoid of C polysaccharide, a common impurity in pneumococcus-derived material that is a highly reactive immunogen resulting in an undesired antibody response (5). Previously, it has been shown that *L. lactis* can produce pneumococcal serotype 3 polysaccharide (3). In contrast, most other known serotypes (2) are synthesized by a relatively simple mechanism. Here we chose serotype 14 as a model for a complex polysaccharide for which biosynthesis occurs via the formation of lipid-linked repeat units prior to their polymerization and export to form capsular polysaccharides.

For the expression of type 14 polysaccharide (PS_{14}) in *L. lactis*, the conserved cassette-like organization of polysaccharide biosynthesis gene clusters in bacteria is exploited in a combinatorial, cassette-based composite expression approach, in which gene cluster-specific regulatory and polysaccharide synthesis cassettes can be exchanged and expressed independently (Fig. 1; see the supplemental material).

Polysaccharides were determined by size exclusion chromatography combined with multiangle light scattering as described previously (14) and by immunodetection with PS_{14}-specific antiserum obtained from the Statens Serum Institute in Denmark. Nisin-induced cells (4) of *L. lactis* harboring pNZ4230 carrying pneumococcal polysaccharide serotype 14-specific genes (*cpsFGHIJKL*$_{14}$) and pNZ4205 carrying polysaccharide B40-specific genes (*epsABCD*$_{B40}$) produced 25 mg/liter polysaccharide recognized by serotype 14 antibodies (Table 1), which is approximately 25% of the level of B40 polysaccharide produced by *L. lactis* harboring pNZ4220 (*epsEFGHIJKL*$_{B40}$) and pNZ4205 (*epsABCD*$_{B40}$). Notably, no polysaccharide was produced by the *L. lactis* strain harboring the pneumococcal *cpsBCDE*$_{14}$ (pNZ4237) genes (Table 1) in combination with pNZ4230 (*cpsFGHIJKL*$_{14}$), while *cpsBCDE*$_{14}$ (pNZ4237) could promote B40 polysaccharide (PS_{B40}) biosynthesis (Table 1) in *L. lactis* harboring pNZ4220 (EPS_{B40}-specific gene cassette).

The PS_{14} produced in *L. lactis* displayed a relatively monodisperse mass range (polydispersity index [M_w/M_n], 1.39) that is typically seen in lactococcal polysaccharides (17) and centered around a mass of 3.1×10^5 Da. The size of the serotype 14 polysaccharide is comparable to the size of the extracellular polysaccharide B40 (EPS_{B40}) (3.3×10^5 Da) produced in *L. lactis* (pNZ4220 and pNZ4205) and is in the same order of magnitude as the commercially available serotype 14 polysaccharide (measured size, 8.6×10^5 Da) that was purchased from the ATCC (American Type Culture Collection, Manassas, VA; ATCC number 197-x). This parameter is relevant since polysaccharide length has been shown to affect immunogenicity, and increased antibody titers against PS_{14} were reported for rabbit models by using polysaccharide conjugates of higher molecular weights (6). Polymer size in *L. lactis* is influenced by medium components (8), which suggests that an adjustment in medium composition can be exploited to eventually optimize the immunogenicity of the PS_{14} produced by *L. lactis*.

Interestingly, immunodetection using serotype 14-specific antiserum revealed that the vast majority of the type 14-specific signal was found in the supernatant rather than attached to the cells (data not shown), suggesting that PS_{14} is released in the culture supernatant by *L. lactis*, which is in contrast to the case

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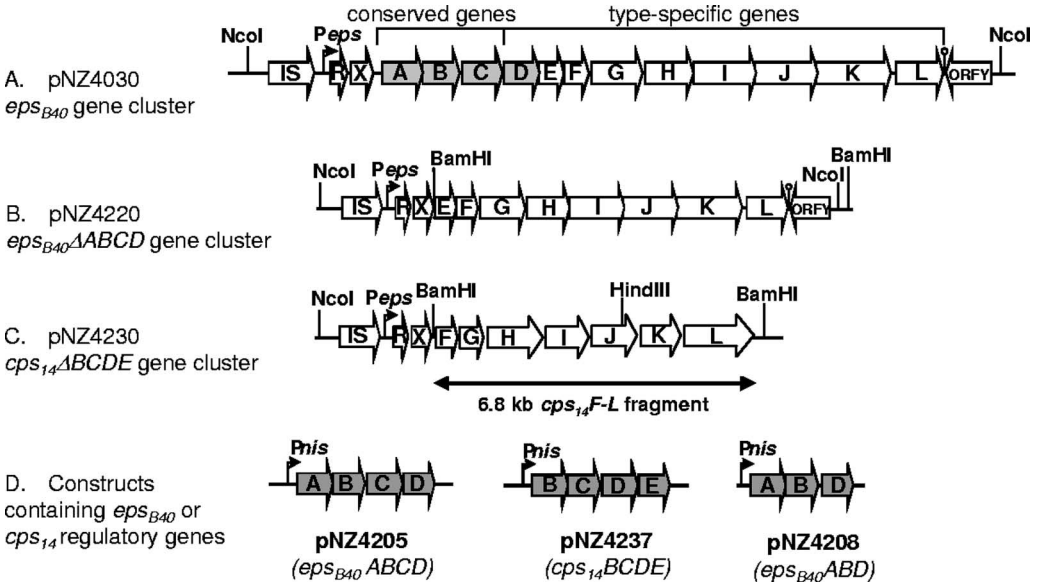

FIG. 1. For the expression of type 14 polysaccharide, we adapted the expression system that was previously shown to be functional for homologous and heterologous expression of *eps* genes in *L. lactis* (14). The type-specific genes from the cps_{14} gene cluster ($cpsFGHIJKL_{14}$) were cloned under the control of the constitutive eps_{B40} promoter on a medium-copy-number plasmid. The conserved regulatory genes as well as the downstream priming glycosyltransferase-encoding gene of the same polysaccharide gene clusters were cloned under the control of the nisin-inducible *nisA* promoter (for pNZ4205, $P_{nisA}epsABCD_{B40}$; for pNZ4237, $P_{nisA}cpsBCDE_{14}$) on a plasmid vector that is compatible with the pNZ4220 and pNZ4230 replicon (18). (A) eps_{B40} gene cluster of plasmid pNZ4030 and P_{eps} promoter of the eps_{B40} gene cluster. (B) In-frame deletion of $epsABCD_{B40}$ from pNZ4030, excision of the resulting gene cluster by NcoI digestion, and ligation into NcoI-digested pIL253 results in pNZ4220. (C) Excision of the $epsEFGHIJKLorfY_{B40}$ genes by BamHI digestion and replacement with a 6.8-kb fragment encompassing $cpsFGHIJKL_{14}$ results in pNZ4230. The HindIII restriction site used for cloning of the PCR-amplified cps_{14} genes is indicated. (D) Plasmids containing the eps_{B40} (pNZ4205) and the cps_{14} (pNZ4237) regulatory genes under the control of the nisin-inducible promoter (4) and a derivative construct (pNZ4208) used in this study (see the supplemental material for genetic construction details).

with its native production host (*Streptococcus pneumoniae*), wherein most polysaccharide is covalently linked to the cell wall (16). This finding could simplify downstream processing aiming to isolate the polysaccharide from culture broth and avoids undesired contaminations when *L. lactis* is used as production host.

We have previously demonstrated that the phosphorylation of $EpsB_{B40}$ in *L. lactis* prevents or strongly reduces EPS biosynthesis (14). Contrary to this result, in *S. pneumoniae* a strong phosphotyrosine-specific CpsD signal was always observed in wild-type cells that produced maximum CPS levels (1, 10, 11). A phosphotyrosine-specific signal was detected in *L. lactis* harboring the PS_{14}-specific genes (pNZ4230) in combination with pNZ4237 ($cpsBCDE_{14}$) (Fig. 2, lane 3), but not in combination with pNZ4205 ($epsABCD_{B40}$) (Fig. 2, lane 1), which is in agreement with the reported production-stimulatory role of the unphosphorylated form of these regulatory proteins in *L. lactis* (14). A regulatory gene cassette lacking the predicted tyrosine phosphatase-encoding gene in the $epsABCD_{B40}$ cassette ($\Delta epsC_{B40}$) (Fig. 1) was constructed (pNZ4208 [14]). No tyrosine-phosphate signal was detected in PS_{14}-producing *L. lactis* expressing the $epsABD_{B40}$ genes

TABLE 1. Polysaccharide isolated from the culture supernatant of *L. lactis* strains harboring *eps* and *cps* gene cassettes

Gene cassette[a]	Polysaccharide (mg/liter)[b]		Polysaccharide at OD_{600} (mg/liter)[c]	Polymer type produced
	Uninduced	Induced		
$\Delta cpsBCDE_{14}$ + $epsABCD_{B40}$ (pNZ4230 + pNZ4205)	<1	25	12	PS_{14}
$\Delta cpsBCDE_{14}$ + $cpsBCDE_{14}$ (pNZ4230 + pNZ4237)	<1	<1		
$\Delta cpsBCDE_{14}$ + $epsABD_{B40}$ (pNZ4230 + pNZ4208)	<1	21	11	PS_{14}
$\Delta epsABCD_{B40}$ + $epsABCD_{B40}$ (pNZ4220 + pNZ4205)	<1	110	56	PS_{B40}
$\Delta epsABCD_{B40}$ + $cpsBCDE_{14}$ (pNZ4220 + pNZ4237)	<1	31	13	PS_{B40}

[a] Constructs are presented as follows: type-specific gene cassette + regulatory gene cassette (corresponding plasmid designations).

[b] The values presented are averages of at least two independent experiments and varied from the mean by no more than 7%. Cells were induced with 1 ng/ml nisin as described in Materials and Methods.

[c] PS production corrected for the final optical density at 600 nm (OD_{600}).

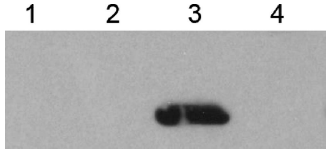

FIG. 2. Tyrosine phosphorylation of EpsB or $Cps_{14}D$ proteins in PS_{B40}- and PS_{14}-producing *L. lactis* strains. Cell extracts of *L. lactis* harboring pNZ4230 in combination with pNZ4205 (lane 1), pNZ4208 (lane 2), and pNZ4237 (lane 3, 4). Cells were either induced (lanes 1, 2, and 3) with 1 ng/ml nisin or uninduced (lane 4). Preparation of cell extracts and detection of the immunoblot analyses were performed as described previously (14) with the following modification: immunodetection was visualized using SuperSignal West Pico chemiluminescent substrate (Pierce) according to the instructions of the manufacturer.

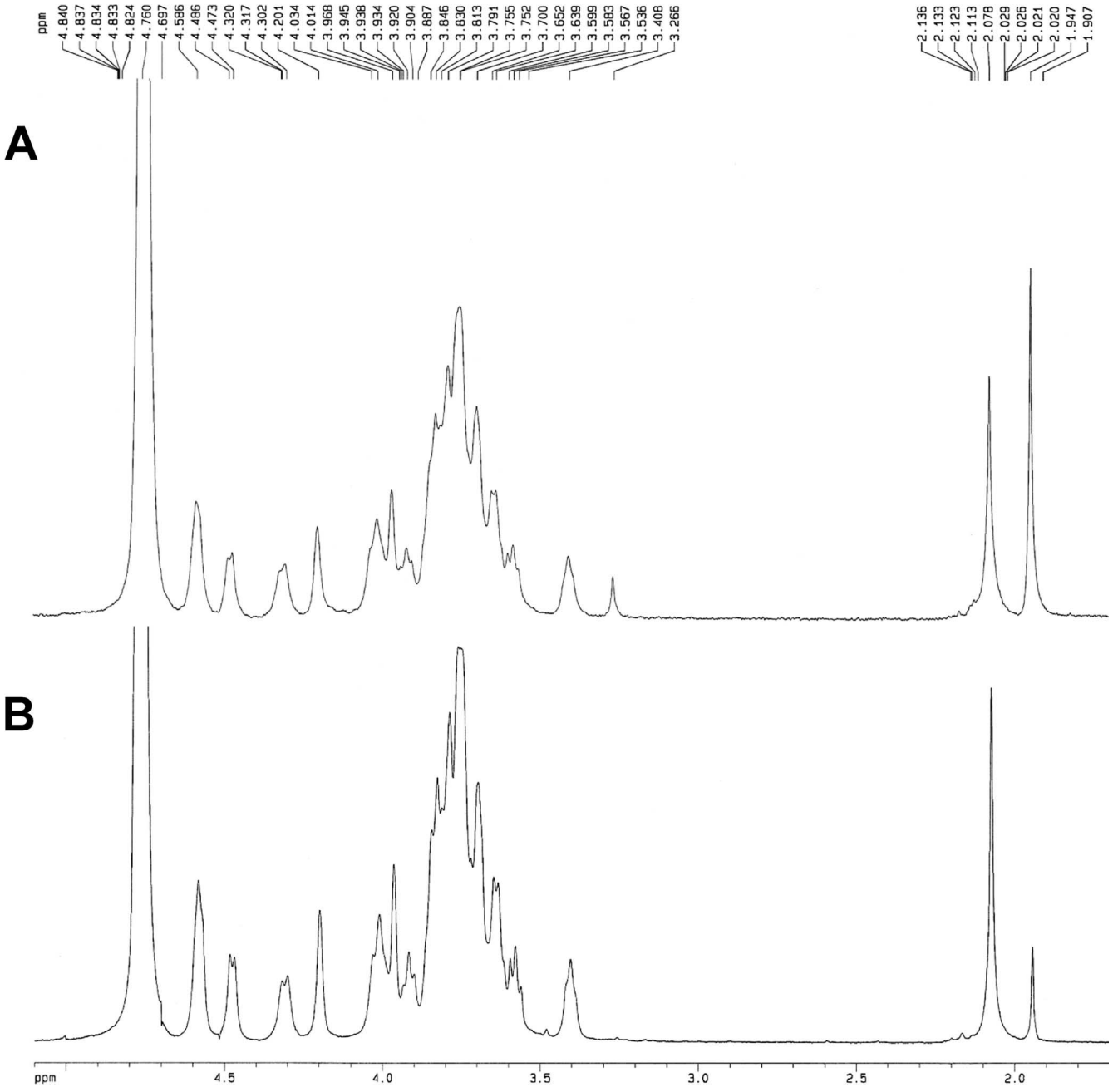

FIG. 3. Proton NMR spectra of PS_{14} purified from *S. pneumoniae* purchased from the ATCC (A) and of PS_{14} purified from *L. lactis* expressing pNZ4230 and pNZ4205 (B).

(Fig. 2, lane 2), indicating that even in the absence of the phosphatase (EpsC), the EpsB in these cells is present in its unphosphorylated form. In addition, this strain still produced PS_{14} at a level that was comparable to that produced in the strain coexpressing $epsABCD_{B40}$ (25 and 21 mg/ml for the $epsABCD_{B40}$ and the $epsABD_{B40}$ constructs, respectively), which was secreted in the medium, thereby excluding the possibility that the deletion of *epsC* affects the subcellular location of the polysaccharide produced in *L. lactis*, as demonstrated previously for *S. pneumoniae* (11). These findings support previous data that indicate that polysaccharide biosynthesis in *L. lactis* is stimulated by the unphosphorylated form of these tyrosine-containing regulatory proteins and suggest a pivotal role of host-specific protein phosphorylation status in polysaccharide production control.

To further establish the identity of the serotype 14 polysaccharide produced in *L. lactis*, comparative proton-nuclear magnetic resonance (NMR) analysis was performed with the polysaccharides isolated from the *L. lactis* culture and a commercially available, serotype 14 polysaccharide purified from *S. pneumoniae* (ATCC). To this end, the polysaccharide-containing solutions were fractionated by size exclusion chromatogra-

phy as described previously (14) and PS_{14}-containing fractions were collected, dialyzed against Millipore water, and lyophilized. Lyophilized samples were dissolved in 99.9% D_2O, and NMR spectra were taken at 400 MHz. The proton-NMR spectra obtained with the PS_{14} produced in *L. lactis* and the ATCC product appeared to be virtually identical (Fig. 3). The minute variation in the two spectra is due to a small impurity present in the PS_{14} isolated from *S. pneumoniae*; this impurity resulted in an additional peak at 3.27 ppm that could not be assigned to any moiety of the published repeating unit (7). These data, combined with the immunodetection data, indicate the chemical identity of the repeating unit present in the polymer produced in *L. lactis* and that of the native production host, *S. pneumoniae*.

S. pneumoniae CPS production levels reported in the literature are 0.5 mg/10^9 CFU (serotype 19F [10]) and 0.6 mg/10^9 CFU (serotype 15B [15]). The PS_{14} level produced in *L. lactis* was estimated to be 0.15 mg/10^9 CFU (corresponds to 25 mg/liter), which is in the same order of magnitude as the reported values for serotype 19F and serotype 15B in *S. pneumoniae*. It should be noted that *L. lactis* was grown in batch cultures without pH control or optimization of fermentation to increase biomass yields. Therefore, improved *L. lactis* cultivation conditions most likely will allow a further increase of polysaccharide production yields relative to those described here.

Overall, *L. lactis* appears to be an attractive, alternative, heterologous production host for polysaccharides of pneumococcal origin but could possibly also be exploited for the production of polysaccharides derived from other gram-positive pathogens. The strategy employed here indicates that maintaining host-derived production control functions allows the overruling of regulatory constraints that might result from the transfer of polysaccharide production and its control from one host to the next.

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