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Pseudomonas aeruginosa PAO1 Ceases To Express Serotype-Specific Lipopolysaccharide at 45°C

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Most *Pseudomonas aeruginosa* strains are able to produce two distinct lipopolysaccharide (LPS) O-polysaccharide types, A-band (common-antigen) and B-band (serotype-specific) LPSs. The relative expression levels of these two LPS types in *P. aeruginosa* PAO1 (O5 serotype) at various growth temperatures were investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining or Western blotting (immunoblotting) with monoclonal antibodies specific for each O polysaccharide. A-band and B-band LPSs were expressed concurrently when the cells grew at 15, 25, and 35°C; however, growth at 45°C resulted in a surface deficiency in B-band LPS as determined by immunoblotting and agglutination with B-band-specific monoclonal antibody. Transfer of these cells (expressing A-band LPS but deficient in B-band LPS) [A^+B^-]) to a lower temperature (at which the division time was comparable) resulted in a rapid resumption of normal A-band and B-band expression. B-band LPS was detectable by immunoblotting before measurable growth of the culture had occurred.

Pseudomonas aeruginosa is ubiquitous in the environment and is also an important human opportunistic pathogen. As such, this organism must be able to readily adapt to widely varying nutrient levels and temperatures. The thermoadaptation of *P. aeruginosa* is complex but appears to involve alterations to both the lipid A and O-antigen components of lipopolysaccharide (LPS) (9, 15). In common with many other gram-negative species, growth at higher-than-optimal temperatures prompts a decrease in the proportion of high-molecular-weight (high-MW) O antigen present on the surfaces of cells (1, 9, 15, 18). It is now recognized that the majority of *P. aeruginosa* strains (including PAO1) possess the ability to produce two distinct O-polysaccharide types (12, 19, 20). The high-MW B band defines the serotype of the strain, with the PAO1 (O5 serotype) subunit consisting of two uronic acid derivatives and one *N*-acetylfucosamine residue (8). The A band contains shorter chains composed primarily of a polymer of α1→2-, α1→3-, α1→3-linked D-rhamnose and low levels of 2-keto-3-deoxyoctulosonic acid (2). With monoclonal antibodies (MAbs) to these LPS types (11, 12), we have been able to further investigate the temperature dependency of A-band and B-band polysaccharide expression in *P. aeruginosa.*

P. aeruginosa PAO1 (H103) was used throughout the present study (4). Cells were cultured in tryptone soy broth at 15, 25, 35, or 45°C with shaking (150 rpm) to stationary phase (ca. 16 h), and 1 ml of culture was then transferred to fresh medium that was maintained at 15, 25, 35, or 45°C. Incubation was continued, and the cells were harvested when the optical density at 470 nm (OD_{470}) of the culture reached ca. 1.0. Before preparation for LPS analysis, samples were standardized with respect to protein by using a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) such that each 10 μl of sample contained 50 μg of total cell protein before digestion. LPS was prepared by the proteinase K method described by Hitchcock and Brown (5) and was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% (wt/vol) acrylamide gels (13). The separated LPS bands were silver stained according to the method described by Tsai and Frasch (21); however, bands were developed with Bio-Rad silver stain developer (Bio-Rad Laboratories, Hercules, Calif.) as described by Petter et al. (17). When required, separated LPS samples were transferred from SDS-PAGE gels to nitrocellulose at 100 V for 1 h, were blocked with 3% (wt/vol) skimmed milk, and were reacted with MAb MF 15-4 (specific for B-band serotype O5 LPS [11]) or MAb N1F 10 (specific for A-band LPS [12]). The bands were visualized with goat anti-mouse antibodies conjugated to horseradish peroxidase, which was developed with 4-chloro-1-naphthol.

For agglutination testing, a thick washed-cell suspension was prepared and 40 μl was placed on a glass microscope slide. A 40-μl drop of MF 15-4 was added to the suspension, and the slide was rocked back and forth for 5 min at room temperature. Visible agglutination within the 5-min period was interpreted as a positive reaction.

Expression of A-band and B-band LPSs in cells cultured at various temperatures. The LPS profiles of *P. aeruginosa* PAO1 cultured at a range of temperatures are shown in Fig. 1. Silver-stained gels (Fig. 1A) showed a characteristic ladder pattern with an uneven distribution of bands. The LPS profiles of cells cultured at 15, 25, and 35°C were essentially the same, and there did not appear to be a drastic change in O-side chain length with increases in temperature as previously noted in other studies (9, 15). However, at 45°C, the banding pattern dramatically changed, especially with respect to the high-MW polysaccharide. Western blotting (immunoblotting) with A-band-specific MAb (Fig. 1B) showed that A-band LPS was present in all samples and that there was a modest increase in the average length of the polysaccharide when cells were cultured at 45°C. Immunoblotting with B-band-specific MAb showed extensive banding in cells cultured at 15, 25, and 35°C; however, cells cultured at 45°C did not express any B-band (serotype-specific) LPS on the surface as determined by this method and failed to agglutinate with B-band-specific MAb, in contrast to cells cultured at 15, 25, and 35°C. During normal growth at temperatures of less than 45°C, *P. aeruginosa* produces membrane vesicles that are excised from the cell surface and which contain only B-band LPS (7). It was possible that if

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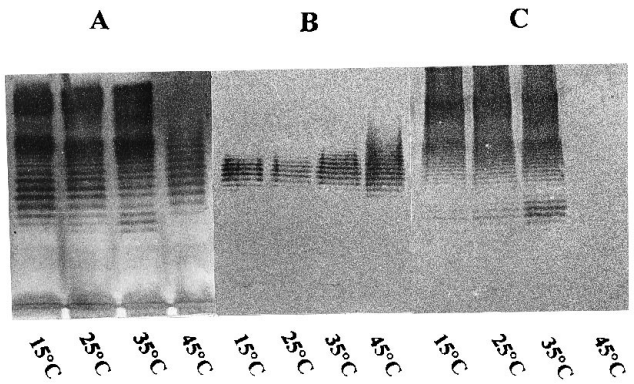

FIG. 1. (A) Silver stained SDS-polyacrylamide gel of LPS from *P. aeruginosa* PAO1 growing at different temperatures. The fractionated LPS was transferred to nitrocellulose and immunoblotted with A-band-specific (B) or B-band-specific (C) MAb.

B-band LPS was still being synthesized, it was being excised from the cells and that it was associated with such vesicles, leaving only the A-band LPS behind. LPS shed into the growth medium was found to be composed entirely of A-band LPS at 45°C, with no B-band LPS detectable by Western immunoblotting, in contrast to growth at lower temperatures, at which both LPS types were detected in the supernatant (data not presented). This strongly suggests that the lack of B-band LPS at 45°C was due to a lack of biosynthesis. These profound alterations in LPS composition are unlikely to be growth rate related, since the division times of cells at 45 and 25°C were essentially the same (ca. 80 min in the present study).

Expression of A-band and B-band LPSs after growth temperature shift from 45 to 25°C. Cells previously grown to stationary phase at 45°C and expressing A-band LPS but deficient in B-band LPS (A^+B^-) were transferred to fresh medium maintained at 45°C, and the medium temperature was brought to 25°C during a period of approximately 10 to 15 min. The culture was incubated at 25°C with vigorous shaking (150 rpm) as described above. At regular intervals, aliquots were removed, and OD_{470} values were determined. In addition, volumes corresponding to 0.5 mg of total cell protein were taken and prepared for LPS. Figure 2A shows a silver-stained gel of samples taken at various times after they were transferred to the lower temperature. The high-MW banding, which was initially absent, rapidly reappears and can be discerned in the silver-stained gel as a smear above the regular banding. Immunoblotting with MAb N1F 10 (specific for A-band polysaccharide) showed that this LPS type was present in all samples, although there appeared to be a small decrease in the average length of this polysaccharide with time (Fig. 2B). Immunoblotting with MAb MF 15-4 (specific for B-band polysaccharide [Fig. 2C]) showed that although it was initially absent at time zero, after only 60 min at the lower temperature, B-band LPS could be detected on the surface, although the major bands had MWs lower than those usually seen. The reappearance of B-band polysaccharide over this short time frame was surprising, since the cells remained in a lag or adaptive phase for at least 120 min after the temperature downshift. If growth had occurred in this time period, there would presumably be division of the oldest cells within the initial population. This was not observed either by viable counting or by optical density measurements, and these data thus strongly suggest that the cells incorporated B-band LPS into their cell envelopes while not in the process of elongation and division. In a similar

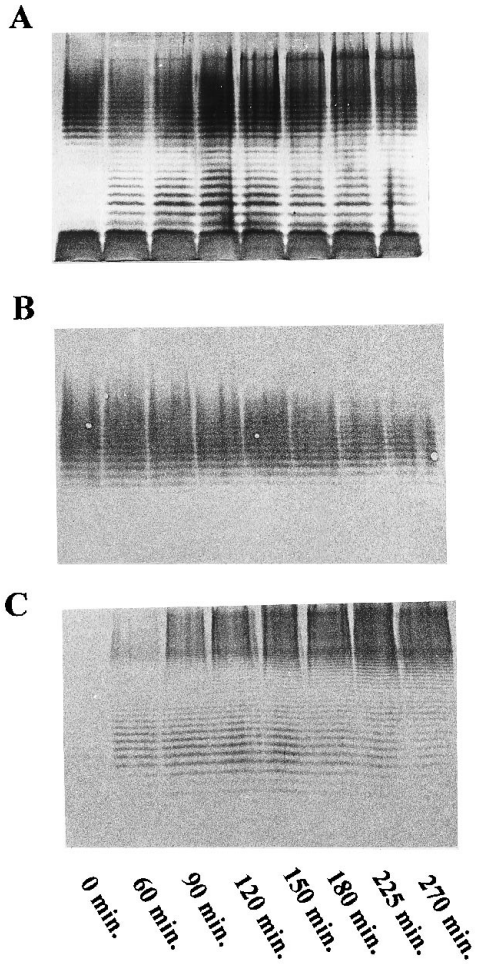

FIG. 2. (A) Silver stained SDS-polyacrylamide gel of LPS from *P. aeruginosa* PAO1 at various times after transfer of cells, which had grown to stationary phase at 45°C, to fresh medium and continued incubation at 25°C. The fractionated LPS was transferred to nitrocellulose and was immunoblotted with A-band-specific (B) and B-band-specific (C) MAb, respectively. It should be noted that growth of the culture was not detectable before 120 min. Time points 150, 180, 225, and 270 min represent 0.38, 0.61, 1.05, and 1.58 generation times, respectively.

experiment, cells grown overnight (16 h) at 25°C were transferred to fresh medium and were cultured at 45°C. There was a gradual loss of B-band LPS, although the conversion to an A^+B^- phenotype tended to take longer (ca. eight generations [data not presented]). This was presumably because the conversion involved shedding (or turnover) of LPS into the growth medium in addition to dilution of the preexisting B-band LPS during cell elongation and division.

In both of the experiments described above, the possibility existed that subpopulations that had an increased ability to grow after temperature shifts were responsible for the observed LPS changes. However, this is unlikely, since the temperature shifts were done over a period of 10 to 15 min and the cold or heat shock responses were thus kept to a minimum. Indeed, when the temperature was increased or decreased in the early exponential phase of growth (OD_{470}, ca. 0.3), very little change in the growth curve was observed and no cell death was seen (data not presented).

The thermoadaptation of *P. aeruginosa* has been shown to involve alterations in the content of unsaturated phospholipid

and LPS fatty acids and outer membrane proteins (3, 9). Kropinski et al. (9) found that when *P. aeruginosa* cells were grown at 45°C, the proportion of S-form LPS decreased and the level of the R form oligosubstituted with side-chain material increased. In a study investigating the influence of the growth environment on the expression of common-antigen and serotype-specific LPSs, McGroarty and Rivera (15) found a decrease in the amount of high-MW B-band LPS in silver-stained gels of LPS isolated from *P. aeruginosa* PAO1716 growing at 42°C. In the present study, we observed that growth at 45°C caused a decrease in the extent of banding found in silver-stained LPS gels and that this was due to the entire absence of B-band LPS, the A-band LPS being conserved (with the occurrence of a moderate increase in the average chain length). We found an extremely rapid LPS response to a decrease in growth temperature, with cells recommencing biosynthesis of serotype-specific LPS even before measurable growth of the culture had occurred.

Temperature affects the molecular motion of LPS, and B-band LPS with its O side chains extending up to 40 nm from the outer membrane surface should become highly unstable as growth temperatures rise. In fact, B-band LPS is the LPS that is preferentially sloughed off from cells (as membrane vesicles) at normal growth temperatures (6). It is possible that at higher temperatures, this type of LPS is incompatible with the high motional attributes of the outer membrane but that when lower growth temperatures are achieved, it can once again be expressed. Within one generation time, cells expressed levels of serotype-specific LPS comparable to those of cells repeatedly cultured at the lower temperature. It is interesting to note that very low-MW bands (i.e., core plus 2 to 6 repeating units) were clearly discernible in immunoblots with B-band-specific MAb MF 15-4 (Fig. 2C). This is in contrast to earlier studies, in which it was postulated that a minimum of 6 to 10 O repeats were required for reactivity with antibodies against serotype-specific LPS (10).

In summary, *P. aeruginosa* cells can profoundly alter the LPS composition of their surfaces very rapidly. We have shown that this happens with temperature shifts; however, such rapid alterations may well occur with other environmental stimuli. Since the relative amounts of A-band and B-band LPSs present on the surface have been shown to affect surface charge, surface hydrophobicity, and susceptibility to antimicrobial agents and host defenses (7, 14, 16), these results may have important implications for all studies involving this organism and, possibly, other gram-negative species.

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