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CDP-Glycerol:Poly(glycerophosphate) Glycerophosphotransferase, Which Is Involved in the Synthesis of the Major Wall Teichoic Acid in *Bacillus subtilis* 168, Is Encoded by *tagF* (*rodC*)

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Assays of CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase (CGPTase) (EC 2.7.8.12) in membranes isolated from *Bacillus subtilis* 168 wild type and 11 strains bearing conditional lethal thermosensitive mutations in *tagB*, *tagD*, or *tagF* revealed that CGPTase deficiency was associated only with mutant *tagF* alleles. In vitro, thermosensitivity of CGPTase strongly suggests that the structural gene for this enzyme is *tagF*. We discuss apparent discrepancies between biochemical evidence favoring a membrane location for TagF and a previous report that suggested a cytoplasmic location based on sequence analysis.

Recent sequencing studies (15) revealed that two adjacent, divergently transcribed operons, *tagABC* and *tagDEF*, are involved (5, 17) in the synthesis of poly(glycerol-phosphate) [poly(groP)], the major cell wall teichoic acid of *Bacillus subtilis* 168. Genes *tagD* and *tagE* encode glycerol-3-phosphate cytidylyltransferase (gro-PCT) (17) and UDP-glucose:poly(glycerol-phosphate) glucosyltransferase (6), respectively. To complete the characterization of the *tagDEF* operon, we have identified the product encoded by *tagF*, in which the majority of known *tag*(Ts) mutations are localized.

B. subtilis 168 strains used are listed in Table 1. Strains containing any of the *tag* mutations described here, when grown at 30°C, synthesize amounts of cell wall teichoic acid comparable to that of the wild type (5). SAT medium and growth conditions were as described previously (5). Membranes (4 to 6 mg) were prepared essentially according to the procedures of Brooks et al. (6) from a 500-ml culture grown at 30°C and harvested in the late-exponential-growth phase (3×10^8 to 4×10^8 cells per ml). Membranes were resuspended in 500 μl of Tris (50 mM, pH 8)–$MgCl_2$ (8 mM)–EDTA (1 mM), divided in 50-μl aliquots, and frozen at −70°C. After 6 months, little activity was lost. Samples were thawed only once, just prior to assay. Protein concentration was measured as described previously (4). Strain L6476 *tagB1*, which accumulates CDP-glycerol (CDP-gro) under growth restrictive conditions (17), was the source of labelled substrate, CDP-[2-^{3}H]gro, required for CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase (CGPTase) assay. After 5 min of labelling with [2-^{3}H]gro (3.2 μM; 260 μCi μmol^{-1}) following transfer of a 200-ml culture to 47°C, the nucleotide fraction, containing generally about 3.5 μCi, was isolated. During initial CGPTase assays, CDP-[2-^{3}H]gro was purified by paper chromatography (17). However, the same incorporation of radioactivity occurred when the crude nucleotide fraction was used. Since a 100-fold excess of unlabelled CDP-gro (50 mM) led to incorporation of negligible amounts of radioactivity, the nucleotide containing eluate was employed as the source of CDP-[2-^{3}H]gro.

CGPTase catalyzes the extension of the main poly(groP) backbone through sequential transfer of glycerol-phosphate units from CDP-gro to an acceptor in the form of growing polyglycerol-phosphate chains attached to the linkage unit lipid (9). The presence of major amounts of such an acceptor in membranes isolated from cells actively synthesizing the cell wall teichoic acid has been demonstrated by their capacity to polymerize precursors of the main teichoic acid chain in the presence of low concentrations of tunicamycin, which blocks the formation of acceptor molecules de novo (2, 9, 13, 19). CGPTase activity in membrane preparations was measured as incorporation of [2-^{3}H]gro from CDP-[2-^{3}H]gro into material retained on a glass fiber filter (6). For economy, CDP-gro was generally used at 0.5 mM, giving approximately half maximal specific activity of CGPTase. Reaction mixtures contained CDP-[2-^{3}H]gro (0.01 to 0.02 μCi), CDP-gro (0.5 mM), UDP-GlcNAc (0.5 mM), $MgCl_2$ (20 mM), membrane protein (50 to 300 μg), and Tris HCl (pH 7.5, 50 mM), in a total volume of 17 μl. Generally, thawed membrane aliquots were pelleted, resuspended in 17 μl of complete reaction mixture, and immediately transferred to a water bath at 30°C. The amount of polymer formed, generally after 15 min, was measured as radioactivity retained on filters, which, after being washed (6), were incubated at 100°C for 30 min in 1 ml of 0.01 M NaOH. Radioactivity was determined by scintillation counting in 10 ml of Optifluor (Packard). Maximal counts, obtained after 3 days, coincided with the filter becoming translucent. In control experiments, incorporation by wild-type membranes, after immersion in boiling water for 2 min, was 1 to 2% of that obtained with untreated membranes.

TABLE 1. Characteristics of *B. subtilis* strains used

Strain[a]	Genotype
L5087	*hisA1 argC4 metC3 tag*$^+$
L6601	*hisA1 argC4 metC3 tagD11*
L6603	*hisA1 argC4 metC3 tagF13*
L6604 through L6608	*hisA1 argC4 metC3 tagF14* through *hisA1 argC4 metC3 tagF18*[b]
L6613	*hisA1 argC4 metC3 tagF19*
L6614	*hisA1 argC4 metC3 tagF20*
L6456	*purA16 leuA8 ilvA1 tagF1* (*rodC1*)[c]
L6476	*hisA1 argC4 metC3 tagB1*

[a] Strains were obtained as described previously (5).
[b] *tag-15*, *tag-17*, and *tag-20* have been mapped to *tagF* (14).
[c] Previous designations include *tag-3* (16, 18), *tagB3* (16), and *tagF3* (15).

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TABLE 2. CGPTase activity in membrane preparations of *B. subtilis* strains carrying mutations in the *tagB*, *tagD*, or *tagF* gene

Mutation	Sp act (nmol/min/mg of protein)[a]						42.5°C/30°C ratio[d]
	At 30°C			At 42.5°C			
	Expt 1	Expt 2	Mutant/*tag*$^+$ ratio[b]	Expt 1	Expt 2[c]	Mutant/*tag*$^+$ ratio[b]	
tagF1	0.00	ND[e]	0.00	0.00	ND	0.00	
tagF13	0.00	0.02	0.01	0.02	0.01	0.01	
tagF14	0.26	0.38	0.15	0.11	0.11	0.08	0.34
tagF16	0.61[f]	0.64	0.29	0.33	0.10	0.07	0.16
tagF17[g]	0.34	0.05	0.09	0.06	0.00	0.00	0.00
tagF18	0.46[f]	0.44	0.21	0.36	0.12	0.08	0.27
tagF19	0.06	0.15	0.05	0.05	ND	0.03	
tagF20	0.18	ND	0.08	ND	ND		
tagD11	2.00	ND	0.90		ND		
tagB1	7.30[f]	7.70	3.40	ND	4.50[f]	3.20	0.60
tag$^+$	1.80[f]	2.60	1.00	ND	1.42[f]	1.00	0.65

[a] Assay conditions are described in the text. Values shown were calculated after deduction of radioactivity incorporated in control experiments with boiled enzyme. 1 and 2 refer to separate experiments with the same membrane preparation. Additional membrane preparations (not presented) of strains carrying *tagD*, *tagB*, and five *tagF* alleles had CGPTase activities similar to those presented.

[b] The ratio of the specific activity of the mutant to that of the wild type.

[c] Membranes resuspended in the incomplete reaction mixture were incubated at 42.5°C for 2 min prior to addition of CDP-gro.

[d] The ratio of the specific activity measured at 42.5°C to that measured at 30°C. The values used come from experiments in which membranes were preheated at 42.5°C.

[e] ND, not done.

[f] In control assays performed in the presence of tunicamycin (1 μg/ml), polymer formation was 90% or more of that obtained in the absence of antibiotic. This reveals that groP incorporation is independent of the formation of de novo acceptor-linkage unit lipid.

[g] Protoplast formation for membrane isolation (6) was extremely and exceptionally slow with this and with another allele, *tagF15* (data not presented); no more than 10% of the cells had formed protoplasts even after 3 h of incubation, and little further change had occurred after 18 h of incubation. It is noteworthy that both mutations map near the N-terminal region of *tagF* (5, 11, 14).

Membrane preparations of the wild type (*tag*$^+$) and mutants, thermosensitive for growth, carrying mutations in the *tagB*, *tagD*, or *tagF* gene (5, 11, 15), were assayed for CGPTase at 30°C, a growth-permissive temperature (Table 2 and data not presented). Unsurprisingly, wild-type activities were associated with mutant alleles of both *tagB*, in agreement with an earlier report (3), and *tagD*, which encodes gro-PCT (17). In marked contrast, nine strains with mutations in *tagF* were all deficient in CGPTase; *tagF1*, *tagF13*, *tagF15* (data not presented), and *tagF19* strains exhibited near zero activity, while the remaining strains, including those with all four mutations, *tagF14*, *tagF16*, *tagF18*, and *tagF20*, associated with a leaky phenotype (5), exhibited activities between 8 and 30% of that of the wild type. Absence of measurable activity in vitro, even at 30°C, in certain mutants may be explained by the unphysiological nature of the assay (7, 17). In additional experiments, the incorporation by membranes of [2-^{3}H]gro for wild-type and *tagB1* alleles, as well as for *tagF16* and *tagF18*, associated with measurable CGPTase activities was insensitive (Table 2) to the presence of tunicamycin (1 μg/ml), confirming that the [2-^{3}H]gro incorporation was independent of the synthesis of a de novo acceptor. To seek further evidence of thermosensitivity of mutated TagF, membranes were assayed at 42.5°C, at which temperature the measured CGPTase specific activity (Table 2) of the wild-type enzyme was reduced, relative to that at 30°C, by about one-third. In comparison, this activity in *tagF* bearing mutants with a leaky phenotype was clearly thermosensitive. Whereas, at 30°C, the relative activities measured as the TagF/Tag$^+$ ratio for *tagF14*, *tagF16*, and *tagF17* mutants are between 9 and 29%, at 42.5°C, they range from 0 to 8%. Thermosensitivity of the CGPTase for three (*tagF16*, *tagF17*, and *tagF18*) out of four mutants examined was revealed more clearly when, prior to assay, membranes were preheated for 2 min at the restrictive temperature (experiment 2, Table 2); this treatment provoked a further three- to fourfold drop in the subsequent incorporation of [2-^{3}H]gro (cf. experiment 1, Table 2).

Results presented provide strong support for concluding that CGPTase is the TagF protein. They complete the characterization of the *tagDEF* operon (15), as well as that of nearly all so-far-identified *tag* mutations. That these enzymes accomplish successive steps in the biosynthetic pathway of teichoic acid (Fig. 1) is fully consistent with coordinate transcription of the respective genes.

To our knowledge, CGPTase is the first identified and sequenced enzyme which catalyzes the polymerization of a diester-linked polyolphosphate chain. The previously reported absence of homology with any other deduced gene product (11), aside from that of *tagB* (15), implies that the deduced sequence of TagF is different from those of other sugar-phosphate polymerases. The homology (15) of the deduced products of *tagB* and *tagF* provides added support for a role for TagB in poly(groP) synthesis, leaving open the possibility that it may recognize polymerized, or monomeric, glycerol-phosphate moieties. Sequence analysis (11) revealed that *tagF* (*rodC*) encodes a protein of 88,063 Da devoid of a signal sequence and with no obvious hydrophobic domains or any other accepted feature characteristic of a membrane protein. However, both chemical and biochemical evidence suggests that TagF is a membrane-bound enzyme. Firstly, its activity, after cell fractionation, is associated with the membrane, and secondly, the polymerization of cell wall teichoic acid is a membrane-linked process involving the participation of the C_{55} isoprenol lipid carrier (1) and the attachment of completed poly(groP) chains to those of nascent peptidoglycan whose synthesis requires the same lipid carrier (10). The suggestion that teichoic acid synthesis is effected by multienzyme complexes, which have been solubilized from *Bacillus licheniformis* (8) and *Micrococcus varians* membranes (13), may offer an explanation for the apparent discrepancy. Thus, proteins encoded by *tag-*

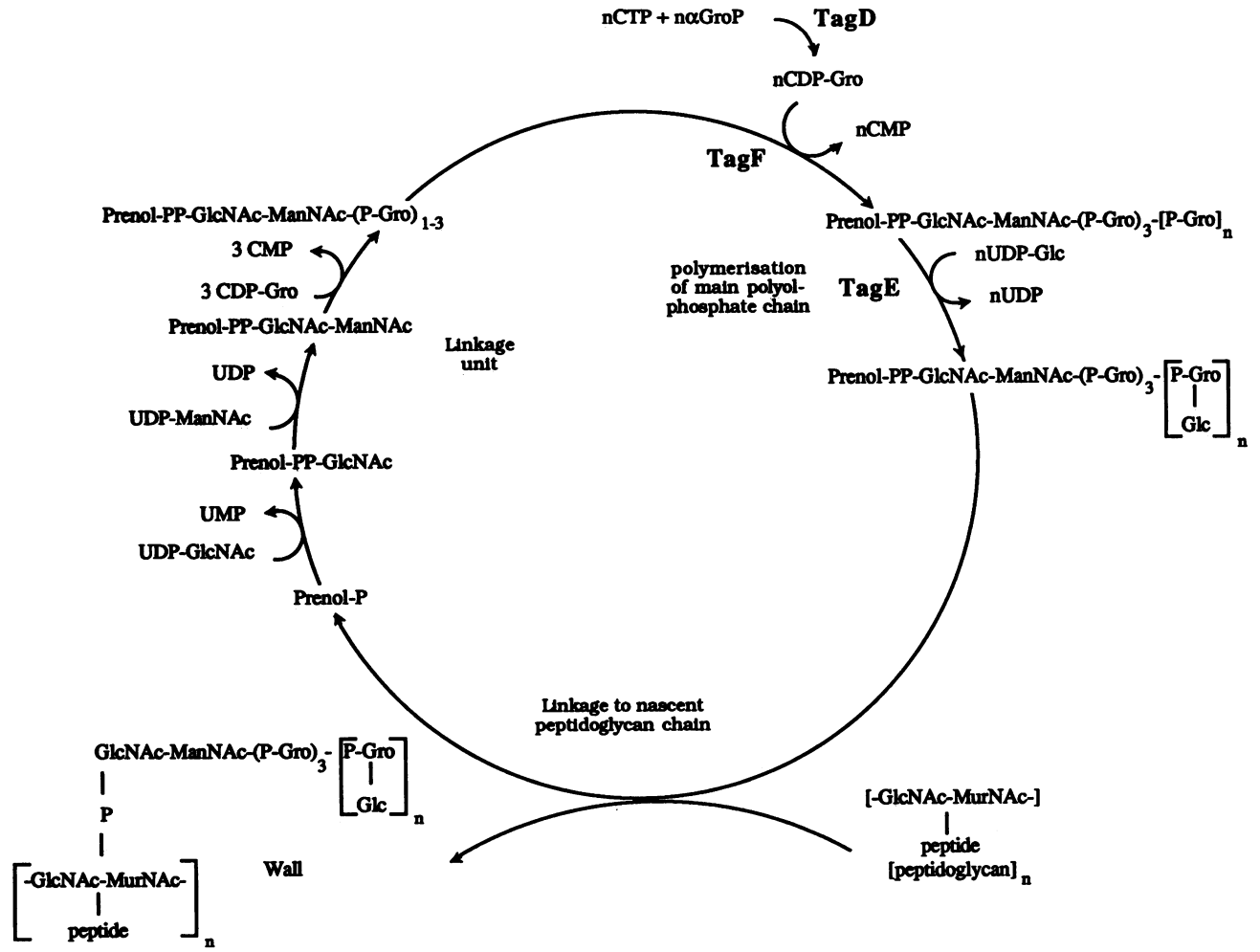

FIG. 1. Participation of products encoded by the *tagDEF* operon of *B. subtilis* 168 in biosynthesis of glucosylated poly(glycerol-phosphate).

DEF and *tagABC* operons may form part of a multienzyme complex, whose association with the membrane could be mediated by just one of the components, for example TagB, which has a hydrophobic domain (15). Interestingly, synthesis of cell wall teichoic acid from exogenously added nucleotide precursors by protoplasts of *B. subtilis* W23 (2) led to the suggestion that relevant enzymes were organized in a transmembrane complex. Equally, in contrast to results obtained with isolated membranes (Table 1), protoplasts of *B. subtilis* 168, as well as of strains carrying *tagF1*, *tagD11*, or *tagB1*, polymerize exogenous CDP-[2-^{3}H]gro at 30 and 47°C (data not presented). Karamata et al. (12) suggested that the phenotypic correction of certain mutations, including *tagF1* (*rodC*), that occurs when intact cells grow in medium with high osmolarity is provoked by the resulting high internal K^+ concentration. If a similar mechanism is responsible for the phenotypic correction of *tagF1* in sucrose-stabilized protoplasts, then an important part of TagF could be located within the protoplast membrane, rather than exposed on the outside. In agreement with this interpretation, no such correction was observed with membranes of *tagF1* or *tagF13* bearing strains assayed in the presence of 0.6 M sucrose or NaCl (data not presented).

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