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NOTES

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***Clostridium perfringens* enterotoxin (CPE) is an important virulence factor for food poisoning and non-food borne gastrointestinal (GI) diseases. Although CPE production is strongly regulated by sporulation, the nature of the signal(s) triggering sporulation remains unknown. Here, we demonstrated that inorganic phosphate (P_i), and not pH, constitutes an environmental signal inducing sporulation and CPE synthesis. In the absence of P_i-supplementation, *C. perfringens* displayed a *spo0A* phenotype, i.e., absence of polar septation and DNA partitioning in cells that reached the stationary phase of growth. These results received support from our Northern blot analyses which demonstrated that P_i was able to counteract the inhibitory effect of glucose at the onset of sporulation and induced *spo0A* expression, indicating that P_i acts as a key signal triggering spore morphogenesis. In addition to being the first study reporting the nature of a physiological signal triggering sporulation in clostridia, these findings have relevance for the development of antisporulation drugs to prevent or treat CPE-mediated GI diseases in humans.**

Clostridium perfringens is a gram-positive, anaerobic, endospore-forming bacterium causing gastrointestinal and histotoxic infections in humans and animals (2, 6, 9, 17). The virulence of this bacterium largely results from its prolific ability to produce at least 15 different toxins (18). In addition, enterotoxigenic *C. perfringens* isolates produce a 35-kDa enterotoxin (*C. perfringens* enterotoxin [CPE]), whose synthesis is under a strict positive control of sporulation (3, 5, 6, 9, 17). In *C. perfringens*, the production of CPE is confined to the large compartment (mother cell) of the sporangium where *cpe* transcription is believed to be driven from the mother cell-specific forms of the RNA polymerase, RNA-σ^E and RNA-σ^K (30). The copious amount of CPE (as much as 10% or more of the total protein of the developing sporangium) is accumulated probably only in the cytoplasm of the mother cell compartment until its release when the mother cell lyses at the completion of sporulation to liberate the mature spore (17). The released CPE rapidly binds to protein receptors present on the apical surface of enterocytes and induces cell permeabilization with the concomitant appearance of the symptoms of enterotoxaemia, intestinal cramping, and diarrhea (2, 17, 18).

Despite the key role of spores in CPE synthesis and in the dissemination and developing of clostridial diseases, very little is known at the molecular level about the regulatory mechanisms governing the formation of spores in clostridia (6, 9, 11, 13, 20, 23). Although from genome sequence analyses it can be assumed that the mechanism of spore formation in *Bacillus* and *Clostridium* is conserved (21, 24, 25), the main differences reside at the level of the initiation of the sporulation process (24, 25). While orthologs for *spo0A* and the genes activated by Spo0A~P, along with most of the *spo* genes that are subsequently expressed during the morphogenesis of the spore, are present in all the sequenced *Clostridium* species, the genes involved in the activation of Spo0A (phosphorelay genes and their regulators) seem to be absent in clostridia (10, 24, 25). The only *spo0* gene found in clostridia is *spo0A*, and therefore it constitutes the unique shared gene of *Bacillus* and *Clostridium* that is clearly involved in the initiation of sporulation in both genera (11, 24).

In this work, we investigated the nature of putative environmental and/or metabolic signals (15) that regulate the commitment of vegetative cells of *C. perfringens* to sporulate and the production of CPE. Examining the growth of *C. perfringens* in Duncan strong sporulation medium (DSSM; 0.4% yeast extract, 1.5% proteose peptone, 0.4% soluble starch, 1% $Na_2HPO_4 \cdot 7H_2O$, and 0.1% sodium thioglycolate) (4), it is possible to appreciate that during the logarithmic phase of growth there is a net decrease in pH that is stabilized with the appearance of mature spores (4 and data not shown). In DSSM, the pH is regulated by the addition of Na_2HPO_4 (inorganic phosphate [P_i]) at a final concentration close to 35 mM. This concentration of P_i in a complex growth medium is unusually high, taking into consideration the nutritional requirement (micromolar amounts) of a bacterial culture for this ion (1, 22, 31). Therefore, one parameter that might regulate

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TABLE 1. Sporulation of *C. perfringens* in DSMM, with or without added phosphate

Added P_i (mM)	Initial pH	Final pH	No. of viable cells/ml	No. of spores/ml	% Sporulation[a]
	6.62	5.04	4.7×10^8	<10	<0.000002
1	6.65	5.05	2.5×10^8	<10	<0.000004
2	6.66	5.05	3.0×10^8	<10	<0.000003
3	6.68	5.07	1.0×10^8	3.2×10^1	0.00003
5	6.73	5.12	3.7×10^7	2.7×10^6	7.29
7	6.89	5.14	1.9×10^7	4.6×10^6	24.21
10	6.91	5.21	1.3×10^7	6.3×10^6	48.46
15	7.03	5.53	4.5×10^7	3.0×10^7	66.66
20	7.17	5.80	3.9×10^7	3.3×10^7	84.61
25	7.29	6.12	1.3×10^7	1.2×10^7	92.30
30	7.35	6.30	2.0×10^7	2.0×10^7	100
35	7.39	6.40	1.4×10^7	1.4×10^7	100
40	7.50	6.53	2.1×10^7	2.1×10^7	100
45	7.55	6.61	3.6×10^7	3.3×10^7	91.66
50	7.59	6.69	4.2×10^7	3.1×10^7	73.80
60	7.61	6.83	8.0×10^6	1.2×10^6	15.00
70	7.68	7.00	2.5×10^6	6.8×10^4	2.72
80	7.80	7.17	9.3×10^5	1.3×10^3	0.14
90	7.93	7.21	4.5×10^5	2.8×10^2	0.06
100	7.98	7.30	3.1×10^5	1.7×10^1	0.005

[a] The percentage of sporulation was calculated as follows: (no. of heat-resistant spores/no. of viable cells) × 100.

the formation of spores in DSSM would be the pH and/or the supplemented P_i.

In order to determine whether P_i and/or pH regulates the capacity of *C. perfringens* to form spores, we grew *C. perfringens* strain SM101 (30) in a modified DSSM (Duncan strong modified medium [DSMM]) supplemented with different concentrations of Na_2HPO_4. As shown in Table 1, at supplemented P_i concentrations of 3 mM or less, the efficiency of sporulation was almost zero. However, the growth of *C. perfringens* was not ameliorated in DSMM without P_i supplementation since the rate of growth was higher in DSMM than that in DSSM (data not shown). Moreover, for the DSMM cultures the exponential phase continued for a couple of hours before reaching the stationary phase of growth in comparison with cultures developed in regular DSSM or DSMM supplemented with 35 mM P_i (data not shown). The final cellular yield was always consistently higher in DSMM (without P_i supplementation) than in DSSM (an average of 4×10^8 CFU/ml versus 2×10^7 CFU/ml) (Table 1). Therefore, the absence of exogenous P_i supplementation in DSMM had no effect on vegetative growth of *C. perfringens* but blocked, during the stationary phase, the differentiation into spores.

In contrast, while 3 mM P_i did not induce sporulation, *C. perfringens* started to sporulate efficiently with the addition of P_i at a final concentration of 5 mM (Table 1). Interestingly, the culture with P_i concentrations between 5 mM and 50 mM did not affect growth and yielded a maximal number of spores (Table 1). Consistent with these results, we detected a high production of CPE after 5 h of growth in DSMM supplemented with exogenous P_i, while no CPE production was detected in non-P_i-supplemented cultures at any stage of growth (Fig. 1). Higher concentrations of P_i (more than 60 mM) reduced the final cellular and spore yields, suggesting some toxic effect of high concentrations of P_i on growth. These results indicated that there is an optimum range of P_i levels for spore formation and suggests, for the first time, that sporulation of *C. perfringens* can be positively regulated by soluble P_i (Table 1).

It can be supposed that P_i did not constitute a nutritional signal because the basal P_i concentration in DSMM, without P_i supplementation, is close to 2 mM, which is at least 100-fold higher than that required for bacterial growth (1, 22, 31). Reinforcing the view that P_i is not a nutritional signal for sporulation, we found that the consumption of P_i after the overnight growth of *C. perfringens* from cultures with or without P_i supplementation was negligible in comparison with the initial concentrations of the anion (data not shown).

In order to distinguish whether the observed effects on spore formation and CPE production were due to the presence of P_i by itself or the regulation of pH by its buffering capacity, we performed an experiment similar to the one described above using DSMM in the absence of P_i supplementation but in the presence of different concentrations of Tris or morpholinepropanesulfonic acid (MOPS) to regulate the pH of the medium (DSMM-Tris or DSMM-MOPS). As observed in Fig. 2, *C. perfringens* cultures grown in DSMM-Tris (or DSMM-MOPS; data not shown) produced similar cellular yields and final pH values as those obtained after growth in DSMM supplemented with different concentrations of P_i. However, under these experimental conditions (growth in DSMM-Tris or DSMM-MOPS without P_i supplementation), *C. perfringens* was unable to sporulate (Fig. 2). Therefore, these results clearly showed that P_i, and not pH, regulated the capacity of *C. perfringens* cells to differentiate into spores.

We then hypothesized that P_i would constitute a universal

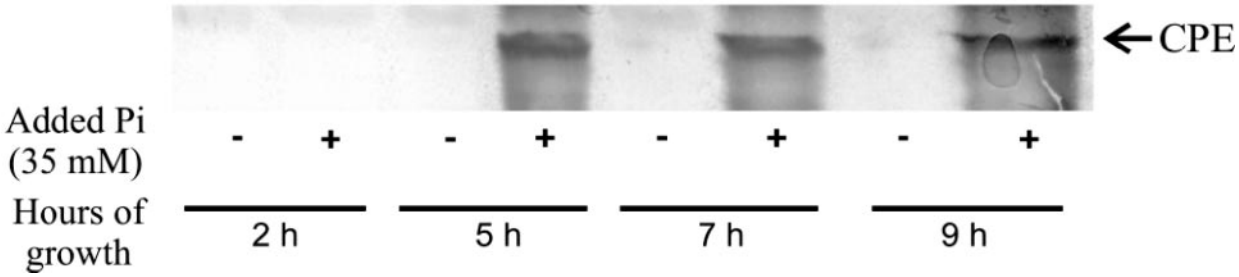

FIG. 1. Western blot analysis of CPE production under P_i-regulated conditions. Cultures of the wild-type strain SM101 were grown in DSMM medium with or without the addition of P_i (35 mM). At the indicated times, samples were removed to be analyzed for enterotoxin production using specific anti-CPE antibodies (11, 20). Control lane (data not shown) containing purified CPE confirmed the identity of the detected band. Results from a representative experiment are shown.

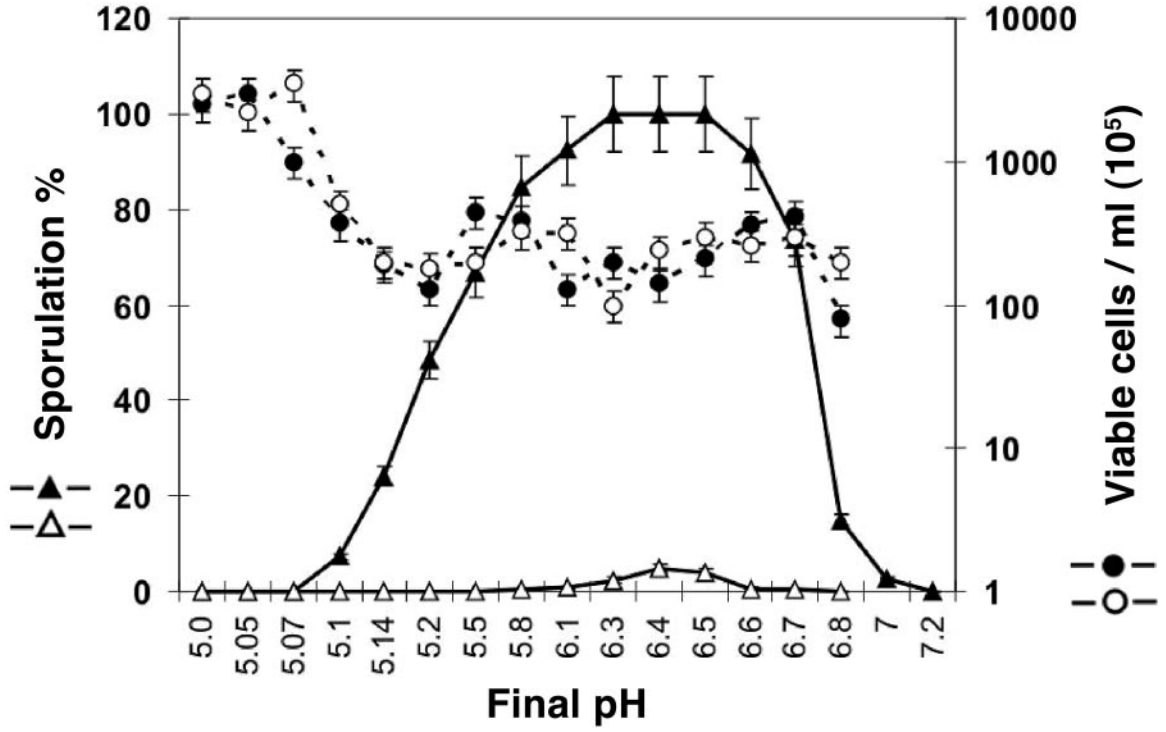

FIG. 2. Efficiency of spore formation under P_i-buffered or Tris-buffered conditions. Cultures of the wild-type strain SM101 were grown in DSMM supplemented with different amounts of 1 M Tris-HCl, pH 8.0, or 1 M Na_2HPO_4 for 20 h at 37°C. The final pH after growth and the final cellular yield (circles) and spore efficiency (triangles) under each growth condition are indicated. Open symbols, Tris-buffered cultures; filled symbols, P_i-buffered cultures. The results shown are the average of five independent experiments; bars indicate standard errors. Similar effects of P_i on spore efficiency were obtained with other enterotoxigenic *C. perfringens* human isolates other than SM101 (data not shown). Essentially the same results as the ones observed in DSMM-Tris were obtained when the Tris buffer was replaced by MOPS buffer (DSMM-MOPS; data not shown).

signal to induce sporulation and CPE synthesis under different growth conditions. To test this hypothesis, we recurred to the use of TGY (tryptone, glucose, and yeast extract) medium and fluid thioglycolate medium (FTG), two rich media commonly used for the vegetative growth of *C. perfringens* where spore formation is completely impaired (3, 11, 20, 30). The intrinsic P_i concentration (<2 mM) in TGY and FTG medium was lower than the threshold P_i concentration (5 to 7 mM) needed to induce spore formation (Table 1). In addition, both media contain high levels of glucose (0.55% and 2.0% for FTG and TGY, respectively) that would induce catabolite repression of sporulation as previously reported (13, 26). In fact, the use of glucose (1%) in DSMM supplemented with 35 mM P_i resulted in a noticeable inhibition of spore formation (data not shown).

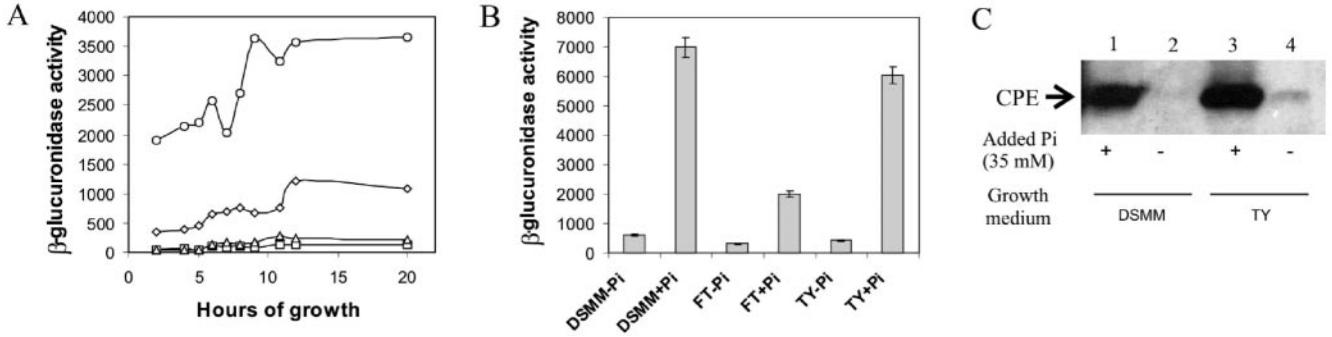

FIG. 3. *cpe* expression and CPE production under different growth conditions. (A) Wild-type cultures of a derivative SM101 strain carrying a *cpe-gusA* plasmid were grown in DSMM with or without the addition of P_i (35 mM) and/or glucose (1%). Samples were removed at the indicated times and analyzed for β-glucuronidase activity as indicated (30). ○, DSMM plus P_i; △, DSMM alone; ◇, DSMM plus P_i and glucose; and □, DSMM plus glucose. (B) Cultures carrying the *cpe-gusA* reporter were developed in three different media able to support the growth of *C. perfringens*: DSMM, TY, and FT medium with or without the addition of P_i (35 mM; see text for details). The bars indicate the level of β-glucuronidase activity accumulated by 1 h after the end of the exponential phase of growth. (C) Western blotting experiment using anti-CPE antibodies (20) of *C. perfringens* cultures grown in the indicated medium with or without supplementation with inorganic phosphate. The cellular pellet from the culture grown in DSMM with P_i was diluted 100-fold, while all the other pellets were undiluted. Data from representative experiments are shown.

TABLE 2. Phosphate-induced sporulation in nonsporulation media

Growth medium[a]	No. of viable cells/ml	No. of spores/ml	Induction (*n*-fold)
DSMM	1.3×10^8	0	>1,900,000
DSMM+P_i	2.0×10^7	1.9×10^7	
FT	3.4×10^7	0	>1,000
FT+P_i	2.0×10^6	1.0×10^4	
TY	3.0×10^7	30	15,333
TY+P_i	2.0×10^6	4.6×10^5	

[a] P_i was used at a concentration of 35 mM.

Furthermore, the expression of *cpe*, measured from a reporter β-glucuronidase fusion (*cpe-gusA*) as previously described (19), in *C. perfringens* cells grown in DSMM supplemented with P_i was strongly repressed in the presence of 1% glucose (Fig. 3A). However, the β-glucuronidase level in DSMM supplemented with P_i and glucose was much higher than the β-glucuronidase activity obtained in non-P_i-supplemented cultures after the addition of glucose (Fig. 3A). In order to avoid the P_i-independent repressive effect of glucose on *C. perfringens* sporulation, we omitted the addition of glucose to the formulation of TGY and FTG. Under these experimental conditions, we observed high levels of sporulation (Table 2), *cpe-gusA* expression (Fig. 3B), and CPE production (Fig. 3C) when glucose-free TGY and FTG (TY and FT, respectively) media were supplemented with exogenous P_i. Collectively, these results indicated that P_i by itself constitutes a universal signal for the sporulation and production of CPE in *C. perfringens*.

In order to determine the precise developmental stage when the presence of P_i is needed for sporulation, we analyzed the cell phenotype of *C. perfringens* cultures grown in DSMM or DSMM-P_i by phase-contrast microscopy as previously described (11). *C. perfringens* wild-type SM101 grown for 5 h in DSMM-P_i showed a great proportion of cells harboring refractile polar prespores (Fig. 4A). In contrast, strain SM101 grown in DSMM (without Pi supplementation) produced cells without any prespores (Fig. 4B). Significantly, the cell phenotype displayed by the non-P_i-supplemented culture of the wild-type cells (Fig. 4B) was indistinguishable from the Spo0$^-$ phenotype displayed by isogenic Spo0A-deficient cells (thus blocked at stage zero) grown in DSMM with or without added P_i (Fig. 4C and D). These results strongly suggested that *C. perfringens* cells grown in non-P_i-supplemented DSMM were blocked at stage zero of the sporulation cycle. This observation was reinforced by fluorescence microscopy analyses using the fluorescent dyes DAPI (4′,6′-diamidino-2-phenylindole; specific for DNA) and FM 4-64 (specific for membrane lipids). For cells grown in P_i-supplemented medium, it was possible to delimit the membrane of the polar prespore (Fig. 4E) with the DNA of the sporangium asymmetrically compartmentalized in both developing cells (Fig. 4G, the prespore and the mother cell). In contrast, for cells from the non-P_i-supplemented cultures, DAPI staining (Fig. 4H) was homogeneous without any asymmetric DNA compartmentalization, while simultaneously the dye for the membrane lipids did not denote any polar membrane (Fig. 4F). These results confirmed that the P_i signal was required at a very early stage of the development of the spore

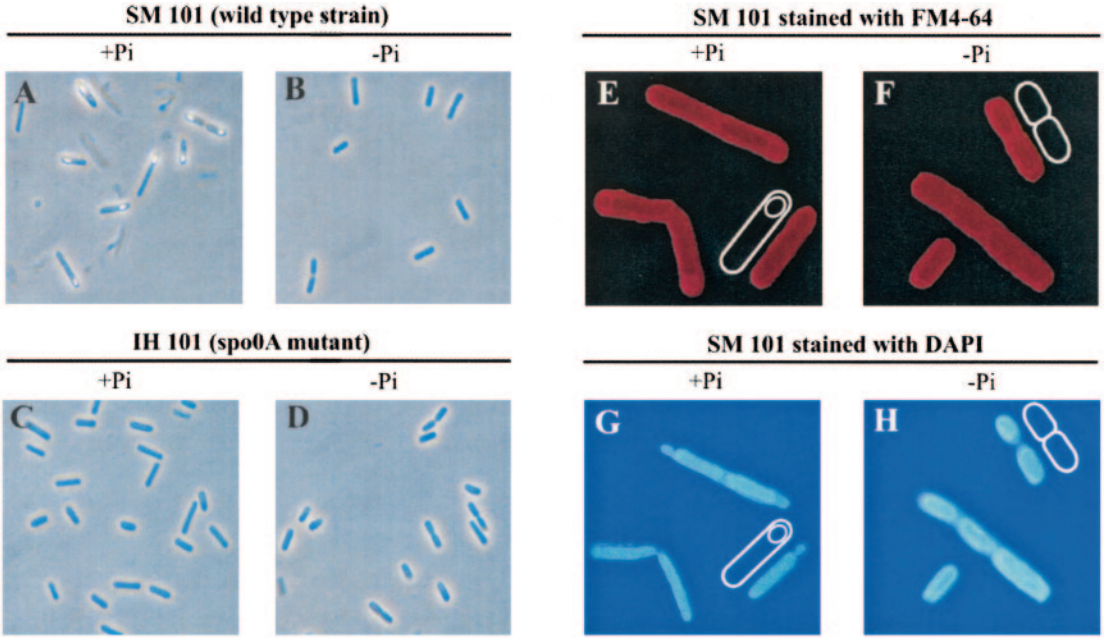

FIG. 4. Cell phenotypes of *C. perfringens* grown in the presence or absence of P_i signaling. (A to D) Phase-contrast photomicrographs of Spo0A-proficient (SM101) and Spo0A-deficient cells (IH101) grown for 5 h in DSMM with or without added P_i. Wild-type cells (SM101) grown in the presence of added P_i displayed polar prespores (A), while the non-P_i-supplemented culture (B) showed cells with a cellular phenotype resembling *spo0A* mutants (11) blocked at stage zero of sporulation, regardless of whether inorganic phosphate was added or not (C and D). After the overnight growth of the wild-type cultures, no prespores were observed in the non-P_i-supplemented culture, while in the P_i-supplemented culture a great proportion of mature and free spores (more than 60%) were observed (data not shown). (E to H) Fluorescent photomicrographs with DAPI (specific for DNA staining) and FM 4-64 (specific for membrane lipid staining) of wild-type cells (SM101) grown for 5 h in DSMM with or without supplementation with P_i.

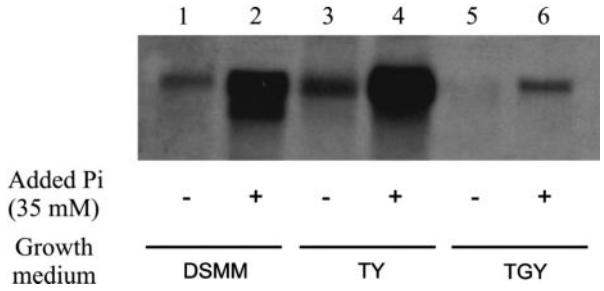

FIG. 5. Phosphate regulates the onset of sporulation in *C. perfringens*. Northern blot analysis of *spo0A* levels under P_i and glucose regulatory growth conditions. Total RNA was extracted and analyzed after 5 h of growth of SM101 cultures in the indicated medium with or without added P_i as previously described (11). The data from a representative experiment are shown.

and strongly suggested that P_i would constitute a sporulation signal acting at the onset of the developmental process before asymmetric division (stage zero).

As indicated earlier, one important requirement for the onset of the sporulation is that sporulation-committed cells induce the expression of *spo0A* (8, 10, 23, 24, 25). Therefore, the level of *spo0A* expression seems to be a valid tool to determine whether a *C. perfringens* culture has initiated the formation of spores beyond stage zero. Northern blotting experiments (11), by detecting the amount of the specific messenger RNA (mRNA) for *spo0A*, confirmed that under conditions of supplementation with exogenous P_i the amount of *spo0A* mRNA far exceeded (20- to 50-fold) the levels of *spo0A* mRNA detected under growth conditions of non-P_i supplementation (Fig. 5, lanes 1 to 4). Thus, these results strongly suggested that P_i acted as a positive signal at the initiation of the sporulation process (stage zero, induced expression of *spo0A*). Furthermore, if this is the situation, P_i should be able to compete with negative sporulation signals acting at the onset of the developmental process (repression of *spo0A* expression). For instance, the blockage of sporulation at stage zero, once a culture has reached the end of the vegetative growth, can be produced by the addition of glucose (catabolite repression of sporulation). In accord with our hypothesis (Fig. 5, lanes 5 to 6), the addition of P_i in a *C. perfringens* culture grown in TGY medium was able (at least partially) to counteract the inhibitory glucose effect on *spo0A* transcription (10, 23, 24, 26) and strongly supports the notion of P_i as a positive environmental signal acting at stage zero of sporulation.

C. perfringens colonizes the small intestine of human and animal where, by unknown mechanisms, it differentiates into spores with the concomitant production of CPE, and then CPE-associated gastrointestinal disorders develop (17). In this regard, one important question arises regarding in vivo significance of P_i as a physiological signal triggering sporulation and CPE production in *C. perfringens*. The intestines represent open environments with plentiful nutrients that support the growth of approximately 500 different bacterial species to the level of 1×10^8 to 1×10^{10} CFU/ml (7). It is possible that sporulation may represent an adaptive response (15) for *C. perfringens* to survive in the stressful environment of the intestine (normal flora, microbicide peptides, bile salts, etc.) rather than a response to a food deficiency, an opposite situation to the regulation of sporulation in *B. subtilis* where unknown signals linked to nutrient starvation induce spore formation (10). It has been estimated from metabolic balance studies that in healthy adults consuming an average Western diet, a P_i concentration of 15 to 30 mM is normally present under homeostatic conditions in the human intestinal lumen (12, 14, 27, 28). This level of in vivo P_i concentration, as we demonstrated in this study, should be able to induce sporulation and CPE production in *C. perfringens*. It is also interesting to note that in all the known P_i-sensing systems reported in bacteria, P_i limitation is the signal that triggers adaptation (29). For instance, the *phoP-phoR* regulatory systems, present in a diverse range of bacteria but apparently absent in *C. perfringens* (21), are activated by depletion of P_i to micromolar levels (1, 16, 22, 31). We demonstrated in this study that an excess amount of P_i, but not P_i starvation, induces the developmental adaptation (sporulation) of *C. perfringens*. Further research on the identification of the signal regulatory system that recognizes millimolar levels of soluble P_i as an environmental signal to induce the initiation of sporulation should help in understanding the mechanism of developmental adaptation of *C. perfringens*.

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