

# Inorganic polyphosphate in *Bacillus cereus*: Motility, biofilm formation, and sporulation

Xiaobing Shi, Narayana N. Rao, and Arthur Kornberg\*

Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305-5307

Contributed by Arthur Kornberg, October 22, 2004

**Chains of inorganic polyphosphate (poly-P) with hundreds of P<sub>i</sub> residues linked by phosphoanhydride bonds, as in ATP, are found in every bacterial, fungal, plant, and animal cell, in which they perform various functions. In the spore-forming *Bacillus cereus*, we have identified three principal enzymes and genes involved in the metabolism of poly-P, namely, (i) poly-P kinase (PPK), which synthesizes poly-P reversibly from ATP, (ii) exopolyphosphatase (PPX), which hydrolyzes poly-P to P<sub>i</sub>, and (iii) poly-P/AMP phosphotransferase (PAP), which uses poly-P as a donor to convert AMP to ADP, reversibly. In the null mutant of *ppk*, poly-P levels are reduced to <5% of the WT; in the *ppx* mutant, the PPK activity is elevated 10-fold, and the accumulation of poly-P is elevated ≈1,000-fold. All of the null mutants of *ppk*, *ppx*, and *pap* showed defects in motility and biofilm formation, but sporulation efficiency was impaired only in the *ppx* mutant. These enzymes and genes in *B. cereus* are nearly identical to those in the very closely related pathogen *Bacillus anthracis*, and, thus, they may provide attractive targets for the treatment of anthrax.**

polyphosphate kinase | exopolyphosphatase | polyphosphate/AMP phosphotransferase

Inorganic polyphosphate (poly-P) is a polymer of tens or hundreds of P<sub>i</sub> residues linked by high-energy phosphoanhydride bonds. Poly-P is ubiquitous, having been found in all organisms (archaea, bacteria, fungi, plants, insects, and mammals) (1–3). Several poly-P-metabolizing enzymes have been purified and characterized, and the corresponding genes have been cloned and deleted (3–8). The enzyme that is primarily responsible for reversible poly-P synthesis in *Escherichia coli* is poly-P kinase 1 (PPK1), which catalyzes the polymerization of the γ phosphate of ATP into a poly-P chain (5). Poly-P can also be hydrolyzed to P<sub>i</sub> either by exopolyphosphatase (PPX) (6) or by endopolyphosphatase (PPN) (7, 8). In *E. coli*, the genes for PPK1 and PPX are under the control of a common promoter. The inability to accumulate poly-P upon deletion of the *ppkppx* operon has produced several striking phenotypes in *E. coli*, including the loss of long-term survival in stationary phase; increased sensitivity to oxidative, osmotic, and thermal stress; and defects in adaptive growth in minimal medium after a shift from a rich medium (9–12). The *ppk1* knockout mutant of *Pseudomonas aeruginosa*, PAO1, shows a dramatic deficiency in motility, an inability to form biofilms, and a loss of virulence (13). Also, poly-P stimulates the kinase activity of mTOR, which is the mammalian target of rapamycin that is involved in the proliferation of mammary cells (14).

*Bacillus cereus*, the spore-forming Gram-positive *Bacillus* found commonly in soil and many other sources, is an opportunistic pathogen that causes food poisoning manifested by diarrhoeal or emetic syndromes. It is related closely to the animal and human pathogen *Bacillus anthracis* (a potential biological weapon) and the insect pathogen *Bacillus thuringiensis* (a pesticide) (15). Recent availability of the genome sequences of *B. cereus* 14579 (American Type Culture Collection) and *B. anthracis* A2012 have revealed a large core set of genes (75–80%) conserved (with 80–100% identity) between these two *Bacilli* species, indicating a common ancestor (16, 17).

Genes that are highly homologous to *E. coli ppk1* and *ppx* and to *P. aeruginosa pap* have been found in *B. cereus*. In this study, these homologues were cloned and overexpressed in *E. coli*, and they were confirmed in their enzymatic activities. Each of the *ppk*, *ppx*, and *pap* genes was knocked out by deletion mutagenesis, and the phenotypes of these mutants are reported here.

## Materials and Methods

**Reagents.** The following reagents were used: ATP, creatine kinase, DNase I, and RNase A (Roche Molecular Biochemicals); [ $\gamma$ -<sup>32</sup>P]ATP and [ $\gamma$ -<sup>32</sup>P]P<sub>i</sub> (Amersham Biosciences); polyethyleneimine-cellulose F TLC plates (Merck); restriction enzymes (New England Biolabs); and common chemicals (Sigma).

**Strains and Plasmids.** *B. cereus* 14579 was obtained from the American Type Culture Collection; *B. cereus* suicide vector pSPUC was a gift from Jo Handelsman (University of Wisconsin, Madison); *B. cereus*/*E. coli* shuttle vector pHT304 was kindly provided by Didier Lereclus (Institut National de la Recherche Agronomique, Paris). The full list of strains and plasmids is given in Table 1, which is published as supporting information on the PNAS web site.

**Media and Growth Conditions.** Cultures were grown in LB or brain–heart infusion (BHI) media; CCY medium (casein hydrolysate/yeast-containing medium) (18) was used to assay sporulation. For poly-P accumulation, a Mops-buffered minimal medium contained glucose (4 mg/ml) as a carbon source and K<sub>2</sub>HPO<sub>4</sub> as the P<sub>i</sub> source. The low-P<sub>i</sub> medium contained 0.1 mM P<sub>i</sub>, and the high-P<sub>i</sub> medium contained 2 mM P<sub>i</sub> (11). Media were supplemented with ampicillin (250 μg/ml), kanamycin (250 μg/ml), spectinomycin (300 μg/ml), tetracycline (10 μg/ml), chlorophenicol (10 μg/ml), or erythromycin (10 μg/ml) for selection of antibiotic-resistant transformants of *B. cereus*.

**Plasmid Construction.** *B. cereus ppk*, *ppx*, and *pap* coding sequences were obtained by PCR using genomic DNA as a template. Each pair of primers yielded a 2.1-kb (for *ppk*), 1.6-kb (for *ppx*), or 0.8-kb (for *pap*) PCR product. For expression in *E. coli*, the PCR fragments were digested with *Nco*I and *Bam*HI and cloned into pTrc99A predigested with the same restriction enzymes. The resulting plasmids were designated as pTrc-BcPPK, pTrc-BcPPX, and pTrc-BcPAP, respectively.

For complementation of null mutants, a low-copy-number plasmid, pHT304, was used (19). The PCR products were digested with *Bam*HI and *Hind*III and cloned into pHT304. The sequences of the primers used are given in Table 2, which is published as supporting information on the PNAS web site.

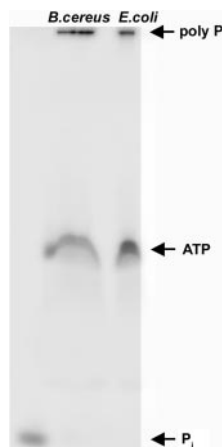
Abbreviations: poly-P, inorganic polyphosphate; PPK, poly-P kinase; PPX, exopolyphosphatase; PAP, poly-P/AMP phosphotransferase; BHI, brain–heart infusion.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database [accession no. BK005415 (*pap*)].

\*To whom correspondence should be addressed at: Department of Biochemistry, Stanford University School of Medicine, B415 Beckman Center, 279 Campus Drive West, Stanford, CA 94305-5307. E-mail: akornber@cmgm.stanford.edu.

© 2004 by The National Academy of Sciences of the USA

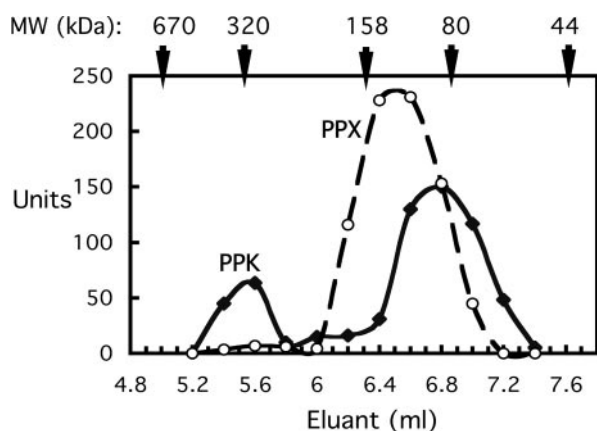




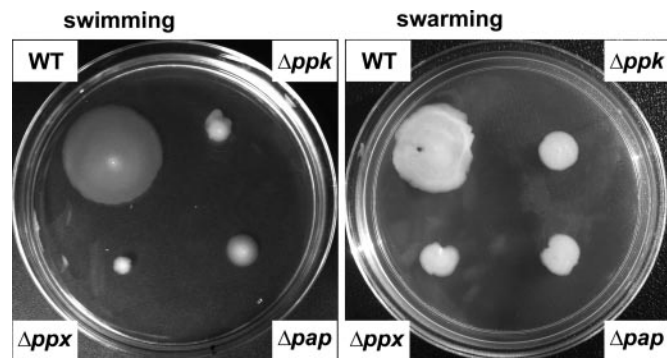
**Fig. 2.** PAGE analysis of poly-P synthesized by *B. cereus* PPK. The products of incubation of [ $\gamma$ - $^{32}$ P]ATP with 40 ng of purified *B. cereus* PPK or 20 ng of *E. coli* PPK at 37°C for 30 min were separated by 20% PAGE containing 7 M urea, and visualized with a PhosphorImager (Molecular Dynamics).

*B. cereus* PPK is processive, producing poly-P with a uniform length ( $\approx 700$ ), similar to the product of *E. coli* PPK1 (Fig. 2).

**Null Mutants of *ppk*, *ppx*, and *pap*.** Null mutants were obtained by replacement of the ORFs of *ppk*, *ppx*, and *pap* with a tetracycline-resistance gene (see *Materials and Methods*). The enzymatic activities of PPK in the  $\Delta ppk$  and PPX in the  $\Delta ppx$  mutant were undetectable ( $<0.1$  units/mg protein). The PPK level in the  $\Delta ppk$  mutant was similar to that in the WT, but remarkably, the PPK level in the  $\Delta ppx$  mutant was  $\approx 10$ -fold higher than that of the WT. However, an inhibitory complex of PPX with PPK was not observed in the WT cell lysates on size-exclusion chromatography. On a Tosoh G2000SW-XL HPLC column (Amersham Biosciences), PPK behaved as a monomer of 80 kDa, but in the presence of  $P_{15}$  or  $P_{65}$  (2 mM in  $P_i$  residues; Sigma), approximately one-fifth of the PPK migrated as a tetramer of 320 kDa. PPX appeared to be a dimer of 130 kDa, whether or not poly-P was present (Fig. 3).



**Fig. 3.** Size-exclusion chromatography of PPK and PPX. Cell lysates of WT cells were prepared as described in HEDSP running buffer (50 mM Hepes-KOH, pH 7.6/150 mM  $Na_2SO_4$ /2 mM  $MgCl_2$ /1 mM EDTA/1 mM DTT/2 mM poly- $P_{15}$ ) (Sigma). After incubation for 30 min on ice, 200  $\mu$ l of lysates were applied to a Tosoh G2000SW-XL HPLC column, eluted with HEDSP buffer, and assayed for enzyme activities. Positions of molecular mass standards were thyroglobulin (670 kDa), bovine  $\gamma$  globulin (158 kDa), and chicken ovalbumin (44 kDa). Arrows at 80 and 320 kDa are judged to mark the PPK monomer and tetramer, respectively.



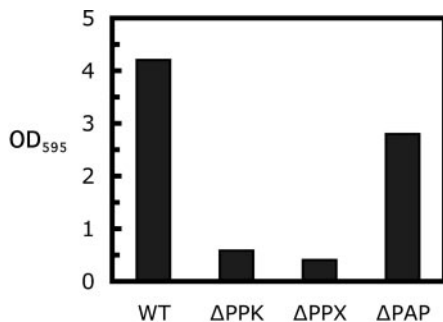
**Fig. 4.** Swimming and swarming motility of WT and the  $\Delta ppk$ ,  $\Delta ppx$ , and  $\Delta pap$  mutants. Cells were inoculated on swimming plates [BHI containing 0.3% (wt/vol) agarose] or swarming plates [BHI containing 0.5% (wt/vol) agar] with an overnight culture (LB agar plates) with a sterile toothpick, and they were photographed after an 8-h incubation for swimming plates or a 14-h incubation for swarming plates at 30°C.

When *E. coli* is downshifted from a high- $P_i$  (2 mM)-Mops-buffered minimal medium to low- $P_i$  (0.1 mM), poly-P accumulated to a level of  $\approx 50$  nmol/mg protein (11). Such a poly-P accumulation was not observed in *B. cereus*. Under the same conditions, WT cells accumulated poly-P to a level of only  $\approx 2$  nmol/mg protein; the  $\Delta ppk$  mutant had  $<5\%$  the WT poly-P level, and the  $\Delta pap$  mutant contained poly-P at a level similar to the WT. Remarkably, the  $\Delta ppx$  mutant accumulated poly-P to a level of 2,600 nmol/mg protein, which is  $\approx 1,000$ -fold that of the WT.

**Impaired Motility in the Mutants.** Deletion of *ppk1* in many Gram-negative strains, such as *E. coli*, *P. aeruginosa*, and *Vibrio cholerae*, impairs their motility (14, 22). In *B. cereus*, flagellar motility observed as swimming and swarming was impaired not only in the  $\Delta ppk$  mutant but even more so in the  $\Delta ppx$  mutant, despite a massive accumulation of poly-P in this mutant (Fig. 4). Deficiency in motility was similar in the  $\Delta pap$  and  $\Delta ppk$  mutants. The motility defects were complemented by introducing the corresponding gene on a low-copy-number plasmid, pHT304 (data not shown).

**Impaired Biofilm Formation in the Mutants.** As in the *P. aeruginosa* *ppk1* mutant (13), mutants of *ppk*, *ppx*, and *pap* in *B. cereus* are each defective in swimming and swarming, as well as in the initiation of biofilm formation. Static biofilm formation was measured by crystal violet staining of attachment to an abiotic (polystyrene) surface. After a 12-h static incubation, only  $\approx 14\%$  of the WT level remained in the  $\Delta ppk$  mutant, 10% in the  $\Delta ppx$  mutant, and 67% in the  $\Delta pap$  mutant (Fig. 5). The deficiencies were not attributable to impairment of growth, the rates of which were indistinguishable from the WT.

**Impaired Sporulation in the *ppx* Null Mutant.** Confronted by environmental stress as in nutrient depletion, the spore-forming bacteria *Bacillus* and *Clostridium* produce dormant spores by undergoing sporulation. As shown in Fig. 6A, *B. cereus* cells started to sporulate in CCY medium after 9–12 h of growth, and sporulation efficiency reached  $\approx 90\%$  after 24 h and almost 100% after 36 h. The  $\Delta ppk$  and  $\Delta pap$  mutants showed similar sporulation efficiency as the WT. Remarkably, the  $\Delta ppx$  mutant showed a 3-h delay in sporulation, and the efficiency was only  $\approx 60\%$ , even after a 36-h incubation. In the WT cells, PPK dropped immediately with the start of sporulation, and remained at  $\approx 5\%$  of the level of vegetative cells, whereas PPX was unchanged during sporulation (Fig. 6B). Consistent with the



**Fig. 5.** Abiotic surface attachment of WT and mutants. An overnight LB culture (2  $\mu$ l) was inoculated into 200  $\mu$ l of LB in 96-well polystyrene plates and incubated at 30°C without shaking. After a 12-h incubation, the media were removed, each well was rinsed with sterile water, and the surface attachments were stained with crystal violet, extracted, and measured as described in *Materials and Methods*.

PPK activity, the poly-P level in WT cells fell during sporulation and remained at a level of 1.8 nmol/mg protein in the spores (Fig. 6B), which is approximately one-third that of the vegetative cells. The  $\Delta ppx$  mutant accumulated poly-P to  $\approx$ 60 nmol/mg protein, which is a level similar to that of  $\Delta ppx$  vegetative cells.

## Discussion

Previous studies of the enzymes and genes in the generation and use of poly-P in Gram-negative bacteria (e.g., *E. coli* and *P. aeruginosa*) demonstrated the essential roles that poly-P plays in responses to stresses and for survival (2, 3, 9–12). In this study, we have explored the distribution of poly-P and the poly-P

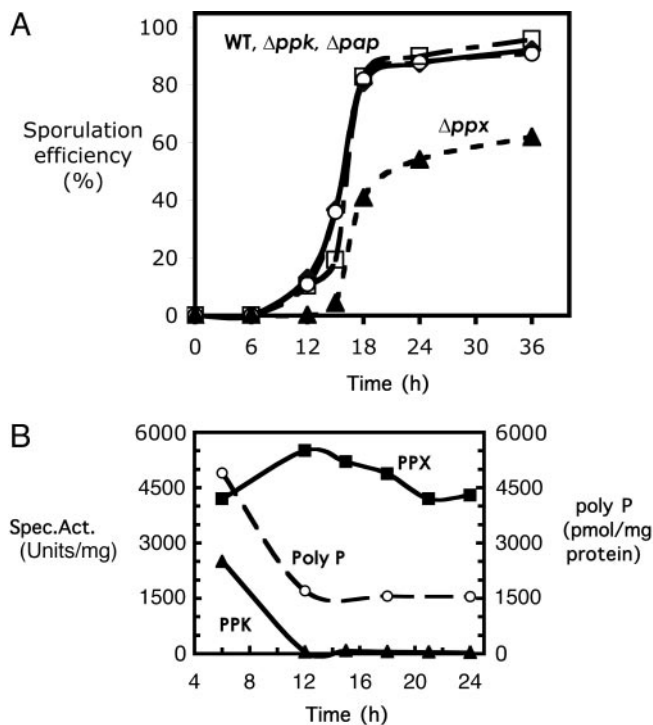
enzymes, genes, and metabolic responses in the Gram-positive, spore-forming *B. cereus*, which is related closely to the serious pathogen *B. anthracis*. PPK, PAP, and PPX and their encoding genes have each been identified and characterized. Null mutants of each gene display phenotypic features that interfere with their behavior and development, such as defective swimming and swarming motilities (Fig. 4) and diminished attachment to an abiotic surface (Fig. 5), which is indicative of poor biofilm development.

Defects in motility are commonly associated with a decrease in virulence (13) as is poor biofilm formation (13, 22). *B. cereus* has alternative forms of flagella-driven motility, such as swimming by individual cells with short and few flagella and swarming by collective swarm cells with long and many flagellae (23). Each of the mutants (*ppk*, *ppx*, and *pap*) showed impairment in both types (Fig. 4). Surface attachment was greatly reduced in the *ppk* and *ppx* mutants but only moderately reduced in the *pap* mutant (Fig. 5). The fact that the *ppx* null mutant showed a more severe defect than the *ppk* mutant indicates that these impairments are likely caused by the fluctuation of the poly-P level in the cell, rather than the alteration of the PPK activity. Maintenance of the intracellular concentration of poly-P within a narrow range may be essential for effectiveness.

The mechanisms by which large fluctuations in poly-P and related enzymes levels produce the various phenotypic features require further study. Phenotype arrays showed that all three mutants had increased sensitivity to the antibiotic reagents miltefosine and alexidine (Table 3, which is published as supporting information on the PNAS web site). Miltefosine is an inhibitor of phosphocholine cytidyl transferase (CTP), which is a key regulator in phosphatidylcholine biosynthesis (24, 25). Alexidine is a cationic antiseptic that inhibits glucosyltransferases which catalyze the formation of glucan from sucrose (28). Electron microscopy showed that mutants grown in a low-phosphate, Mops-buffered medium accumulated phase bright clusters (data not shown), perhaps the result of membrane invaginations during fixation due to the changes in the cytoplasmic membranes or the cytoskelons in the mutants (A. Makhov, personal communication).

Of the several PPKs isolated from *E. coli* (5), *P. aeruginosa* (13), *Salmonella* sp. (29), *Shigella flexneri* (29), *V. cholerae* (22), and *Helicobacter pylori* (3), that of *B. cereus* is the most distinctive. The PPK of *B. cereus* has a low pI, fails to adsorb to anion exchangers, and is stimulated 10-fold in activity in lysates by the presence of poly-P. This stimulation may be due to facilitating the formation or stabilization of a more active oligomer of PPK, as observed with the PPK2 of *P. aeruginosa* (26, 27), or counteracting a high level of PPX activity. In the *ppx* null mutant, PPK activity in the lysates is increased 10-fold, and the accumulation of poly-P in the cell increased 1,000-fold. No such effects have been observed with other *ppx* mutants. The adjacency of *ppk* and *ppx* genes may constitute an operon encoding a PPK–PPX complex. Inhibition of PPK activity by PPX in such a complex might account for the heightened PPK activity and the accumulation of poly-P in the *ppx* mutant. Colocalization of PPK and PPX has been observed in the *E. coli* nucleoid (A. Kuroda, personal communication).

Another distinctive feature of poly-P metabolism in *B. cereus* is the lack of its accumulation in response to a nutritional stringency. Unlike *E. coli* and *P. aeruginosa* in which a switch from a high to a low  $P_i$  level in the medium results in a massive accumulation of poly-P (i.e., 50–100 nmol/mg protein), the response in *B. cereus* is feeble (i.e.,  $\approx$ 2 nmol/mg protein). In *E. coli*, the *ppk* mutant is deficient in adapting to stresses and fails to survive in stationary phase (11). *B. cereus* possesses both a rapid, reversible adaptive stress response that is governed by  $\sigma^B$  and an ability to differentiate into dormant spores under extreme conditions (30–32). Although sporulation did not appear to be



**Fig. 6.** Sporulation of WT and mutants. (A) Sporulation efficiency. Cultures were grown in CCY sporulation medium. Samples at the indicated times were diluted, heated at 70°C for 15 min, and plated on LB agar. Spore frequency was determined by the population that survived the heat treatment and was counted as colonies the next day. (B) PPK, PPX enzymatic activities, and the poly-P level in the sporulating WT cells. Enzyme assays and poly-P determination were performed as described in *Materials and Methods*.

defective in the *ppk* mutant with the assay used, it may prove to be defective under other conditions. For example, sporulation efficiency in the *Myxococcus xanthus ppk* mutant is decreased when induced by glycerol, and in *Dictyostelium discoideum*, the *ppk1* mutant shows a marked delay in fruiting body formation that precedes sporulation (H. Zhang, personal communication). Remarkably, the *ppx* mutant of *B. cereus* showed a decreased sporulation efficiency; in spores of the mutant, poly-P accumulated up to 60 nmol/mg protein, >30-fold than in the WT spores. The lack of poly-P accumulation in response to a nutritional stringency in WT *B. cereus* and the defects in sporulation of the *ppx* mutant accompanied with poly-P accumulation in its spores indicate important roles that poly-P plays in development. In the WT cells, PPK and poly-P levels dropped immediately with the start of sporulation, whereas the PPX level remained unchanged (Fig. 6). Loss of poly-P in the cell triggers the start of sporulation, where Spo0A, the key regulator for initiation of sporulation may be the target of poly-P regulation in the nucleoid.

The fact that PPK can serve as a target for screening antibiotics has already been demonstrated for PPK1 in *P. aeruginosa* (S. Lee, personal communication); PPX and PAP now provide additional targets. Recent availability of genome sequences of *B. cereus* and *B. anthracis* has revealed that a large core set of genes is conserved between these two species (17, 18). The defects in the virulence-related phenotypes of null mutants of *ppk*, *ppx*, and *pap* genes and the very high homologies of the corresponding proteins in *B. anthracis* (97–98% similarity) are attractive targets for discovery of antibiotics for the treatment of anthrax.

We thank Dr. Michael Brown and Leroy Bertsch for advice and assistance in preparation of this manuscript, Dr. Jo Handelsman for plasmid pSPUC and technical suggestions for making knockout mutants, Dr. Didier Lereclus for plasmid pHT304, and Dr. Bill Burkholder (Stanford University) for plasmid pDG1515. This work was supported by the National Institutes of Health.

1. Kulaev, I. S. (1979) in *The Biochemistry of Inorganic Polyphosphate* (Wiley, New York).
2. Kornberg, A. (1995) *J. Bacteriol.* **177**, 491–496.
3. Kornberg, A., Rao, N. N. & Ault-Riché, D. (1999) *Annu. Rev. Biochem.* **68**, 89–125.
4. Wood, H. G. & Clark, J. E. (1988) *Annu. Rev. Biochem.* **57**, 235–260.
5. Ahn, K. & Kornberg, A. (1990) *J. Biol. Chem.* **265**, 11734–11739.
6. Akiyama, M., Crooke, E. & Kornberg, A. (1993) *J. Biol. Chem.* **268**, 633–639.
7. Kumble, K. & Kornberg, A. (1996) *J. Biol. Chem.* **271**, 27146–27151.
8. Sethuraman, A., Rao, N. N. & Kornberg, A. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 8542–8547.
9. Rao, N. N. & Kornberg, A. (1996) *J. Bacteriol.* **178**, 1394–1400.
10. Shiba, T., Tsutsumi, K., Yano, H., Ihara, Y., Kameda, A., Tanaka, K., Takahashi, H., Munekata, M., Rao, N. N. & Kornberg, A. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 11210–11215.
11. Rao, N. N., Liu, S. & Kornberg, A. (1998) *J. Bacteriol.* **180**, 2186–2193.
12. Ault-Riché, D., Fraley, C. D., Tzeng, C. M. & Kornberg, A. (1998) *J. Bacteriol.* **180**, 1841–1847.
13. Rashid, H., Rumbaugh, K., Passador, L., Davies, D. G., Hamood, A. N., Iglewski, B. H. & Kornberg, A. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 9636–9641.
14. Wang, L., Fraley, C. D., Faridi, J., Kornberg, A. & Roth, R. A. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 11249–11254.
15. Helgason, E., Okstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. & Kolsto, A. B. (2000) *Appl. Environ. Microbiol.* **66**, 2627–2630.
16. Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapratral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A., et al. (2003) *Nature* **423**, 87–91.
17. Read, T. D., Peterson, S. N., Tourasse, N., Baillie, L. W., Paulsen, I. T., Nelson, K. E., Tettelin, H., Fouts, D. E., Eisen, J. A., Gill, S. R., et al. (2003) *Nature* **423**, 81–86.
18. Stewart, G. S., Johnstone, K., Hagelberg, E. & Ellar, D. J. (1981) *Biochem. J.* **198**, 101–106.
19. Arantes, O. & Lereclus, D. (1991) *Gene* **108**, 115–119.
20. Dunn, A. K., Klimowicz, A. K. & Handelsman J. (2003) *Appl. Environ. Microbiol.* **69**, 1197–1205.
21. O'Toole, G. A. & Kolter, R. (1998) *Mol. Microbiol.* **28**, 449–461.
22. Ogawa, N., Tzeng, C.-M., Fraley, C. D. & Kornberg, A. (2000) *J. Bacteriol.* **182**, 6687–6693.
23. Senesi, S., Celandroni, F., Salvetti, S., Beecher, D. J., Wong, A. C. & Ghelardi E. (2002) *Microbiology* **148**, 1785–1794.
24. Martinez-Morales, F., Schobert, M., Lopez-Lara, I. M. & Geiger, O. (2003) *Microbiology* **149**, 3461–3471.
25. Sohlenkamp, C., Lopez-Lara, I. M. & Geiger, O. (2003) *Prog. Lipid Res.* **42**, 115–162.
26. Zhang, H., Ishige, K. & Kornberg, A. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 16678–16683.
27. Ishige, K., Zhang, H. & Kornberg, A. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 16684–16688.
28. Venkitaraman, A. R., Vacca-Smith, A. M., Kopec, L. K. & Bowen, W. H. (1995) *J. Dent. Res.* **74**, 1695–1701.
29. Kim, K. S., Rao, N. N., Fraley, C. D. & Kornberg, A. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 7675–7680.
30. Petersohn, A., Brigulla, M., Haas, S., Hoheisel, J. D., Völker, U. & Hecker, M. (2001) *J. Bacteriol.* **183**, 5617–5631.
31. Ireton, K., Rudner, D. Z., Siranosian, K. J. & Grossman, A. (1993) *Genes Dev.* **7**, 283–294.
32. Hamon, M. A. & Lazazzera, B. A. (2001) *Mol. Microbiol.* **42**, 1199–1209.