*Clostridium difficile* causes antibiotic-associated diarrhea in humans, with approximately 250,000 cases and 14,000 deaths per year in the U.S. alone. A Gram-positive obligate anaerobe, the formation of metabolically dormant, oxygen-resistant endospores allows transmission between hosts. Once in the anaerobic large bowel, *C. difficile* germinates, grows and produces cytotoxins that elicit disease symptoms. Little is known about how *C. difficile* senses in vivo signals and initiates its virulence program. In many bacterial species, the nucleotide second messenger cyclic diguanylate monophosphate (c-di-GMP) plays an integral role in physiological adaptation to environmental conditions. C-di-GMP is well known to regulate the switch between motile lifestyles and non-motile forms often involving biofilm formation. Yet this regulatory paradigm was developed primarily based on studies of Gram-negative bacteria; the roles of c-di-GMP in Gram-positive species were not well studied. By manipulating intracellular c-di-GMP levels in *C. difficile*, we have determined that this signaling molecule regulates the production of factors with predicted roles in intestinal colonization, flagella and Type IV pili, and of known virulence determinants, the cytotoxins TcdA and TcdB. These studies suggest that c-di-GMP is poised to control processes fundamental to *C. difficile* disease development.