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DONALD D. CHEN for the degree of Master of Science in Microbiology presented on September 2, 1993

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Dr. Janine E. Trempy

Two chromosomal DNA fragments unique to *Bacillus subtilis* were identified by their ability to encode activities that suppress capsular polysaccharide synthesis in an *Escherichia coli* strain mutant for Lon protease activity. These activities also suppress a second phenotype associated with *E. coli* Δ*lon* mutants - sensitivity to DNA damaging agents (SOS response). These two fragments are not homologous to the *E. coli* gene that encodes the Lon protease, nor are the proteins they encode structurally similar to the Lon protein. The *B. subtilis* activities do not suppress capsule synthesis by repressing expression of the known capsule gene transcriptional activators, RcsA and RcsB, nor do they affect the expression of the Lon substrate, SulA, a participant in the SOS response. The DNA sequences coding for the *B. subtilis* activities correspond to a sequence coding for the *B. subtilis* gluconate operon, and to an open reading frame (ORF5) of unknown function that is located chromosomally next to the pyruvate dehydrogenase operon. Maxicell analysis revealed that one of the DNA fragments expressed a protein comparable in size to the gluconate permease; the other DNA fragment expressed a protein comparable in size to the ORF5 gene product. The mechanisms by which the *B. subtilis* activities suppress capsule synthesis in *E. coli* mutant for Lon protease will be explored.

Regulating Polysaccharide Synthesis in Bacteria

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Typed by Donald D. Chen for researcher.

DEDICATION

This work is dedicated to my parents, Huan-Yung and Suzy Wang Chen, and to the memory of my sister, Julia Ann Chen.

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TABLE OF CONTENTS

LIST OF FIGURES

page

FIGURE

LIST OF TABLES

page

Regulating Polysaccharide Synthesis in Bacteria

INTRODUCTION AND LITERATURE REVIEW

The ability of bacteria to respond to changes in their environment determines how successfully they can develop and grow. It serves no purpose if an organism has developed an aggressive mode for reproduction without a corresponding ability to survive in its present environmental niche. The immediate demands for survival must be met prior to any long term plans for growth and development. In an environment of low temperatures and nutrient deprivation, *Escherichia coli* cells produce a mucoid capsular polysaccharide. This serves to shield this microorganism from a poor environment and allow them to grow and develop.

The types of polysaccharides.

Many bacterial species produce an extracellular polysaccharide layer which may exist associated as a capsular layer or as a more loosely associated mucoid layer. These layers are found in many common bacteria including *Escherichia coli* and *Bacillus subtilis* (45).

Capsular polysaccharides, in the form of stably associated cell constituents, occur in both Gram-negative and Gram positive bacteria. The same holds true for the more loosely associated mucoid layer. Examples of the capsular polysaccharide include the K- and O-antigens of *E. coli*, the streptococcal A, B, and C strain hyaluronic acid, the *Klebsiella* capsular antigens, and the *B. anthracis* poly-D-glutamate (37, 47, 52). Examples of the mucoid capsular polysaccharide include the colanic acid polymer of *E. coli*, the alginate polymer of *Pseudomonas aeruginosa*, and the viscous polysaccharide of *B. circulans* (21, 35, 45).

The capsular layer, found in both Gram-negative and Gram-positive bacteria, is a virulence factor in many pathogenic species, and may offer a measure of protection against environmental conditions and host-associated defense systems (15). In *E. coli*, the capsule antigens (K-antigen) are structurally diverse and lead to serotype specificity. The K-antigens are distinguished by their interference with O-antigen serology (38). They can be distinguished from the neutral O-antigens by their mostly acidic nature and by immune electrophoresis patterns with both anti-O and anti-OK antisera (reviewed in 39). The K-antigens have been further delineated into A-, L-, and B-antigens. The A-antigens in *E. coli* lack amino sugars and exhibit one peak following alkali treatment and analytical ultracentrifugation. L-antigens are associated with the O-antigens and do contain amino sugars. B-antigens also contain amino sugars. In human infections, the wide diversity of *E. coli* capsules elicit over seventy antigenic reactions. In addition to the capsular polysaccharide, the lipopolysaccharide (O-antigen) is another feature of the outer membrane which elicits an antigenic response (37).

In *E. coli*, a mucoid extracellular polysaccharide is also produced. The colanic acid polysaccharide, or M-antigen is a repeating hexasaccharide unit of D-glucose, D-galactose, L-fucose and D-glucuronic acid in ratios of 1:2:2:1 (45). In addition, there are additions of an acetyl group linked to fucose and a pyruvyl group to galactose. (see Figure 1; 21, 45). In *E. coli*, conditions which induce mucoid capsule synthesis include low temperatures and nitrogen limitations while complex media and high temperatures serve as negative stimuli (45).

In *P. aeruginosa*, a mucoid capsular polysaccharide, alginate, is found in clinical isolates from patients diagnosed for cystic fibrosis (14). Both *E. coli* and *P. aeruginosa* can lose the mucoid phenotype by repeated isolations in the laboratory. Unlike the membrane associated polysaccharide, the mucoid phenotype appears to be regulated in response to the external environment. The *P. aeruginosa* alginate is a member of a family of related structures first described in brown algae. The structure is a polysaccharide composed of β-D-mannuronate and α-L-guluronate which are uronic acid epimers at C5 (21).

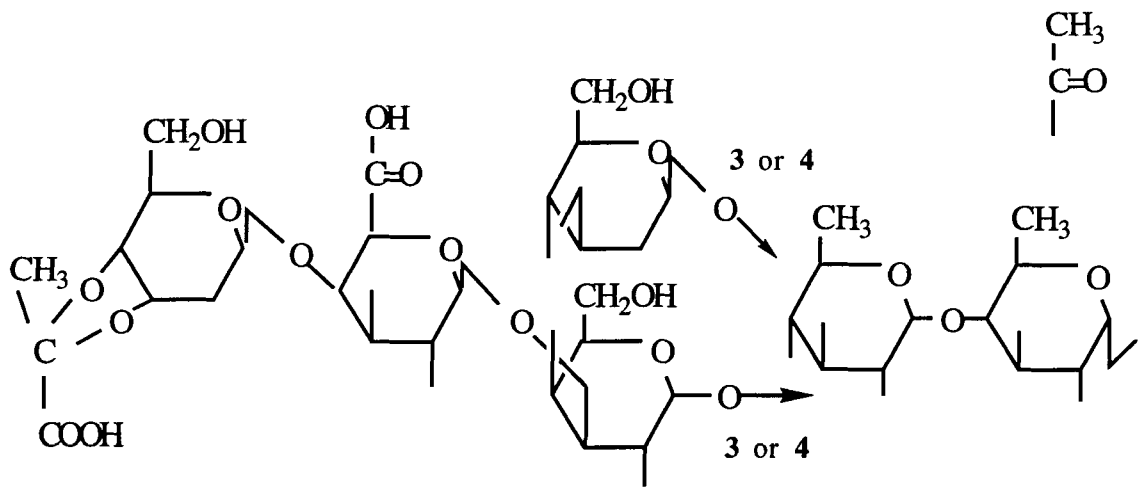

Figure 1. Proposed structure of the colanic acid capsular polysaccharide of Escherichia coli K-12. The hexasaccharide is composed of D-glucose, D-galactose, L-fucose, and D-glucuronic acid in a ratio of 1:2:2:1. There are also additions of an acetyl group to fucose and a pyruvyl group to galactose. The capsule is also known as the M antigen or type I capsule polysachharide. (Figure adapted from Markovitz, 1977.)

In addition, the bacterial alginate is O-acetylated; the degree of O-acetylation is still under investigation with the preliminary finding that the molar ratio of O-acetyl to mannuronate is greater than one.

The roles of polysaccharides.

Polysaccharides offer bacterial organisms a measure of protection from external conditions in the environment or against host defense systems (15).

In *E. coli*, the capsular polysaccharide provides protection from the acidic environment during the passage of enterobacteria through the stomach (15). Streptococcal infections rely on the phagocytic resistance of the hyaluronic acid capsule while lytic enzymes from the organism break down cell walls (77). The *Klebsiella* capsular antigens protect against phagocytosis while the enterotoxin of the organism can induce the hypersecretion of fluids into the lumen of the small intestine (60). In *P. aeruginosa*, the presence of mucoid colonies from sputum samples of cystic fibrosis patients has thought to be the result of a colonization phenotype which prevents dislodgment from pulmonary surfaces by coughing or other physical mechanisms (14).

The production of the mucoid layer can also protect the organism from the natural environment, providing a mechanism of adherence to soil or other structures (15). Formation of the *E. coli* mucoid layer is induced by low temperatures or starvation for nutrients (26). Although the mucoid layer may not always be apparent, it has been proposed that the layer protects the organism when nutrients and proper temperature are unavailable, allowing it to survive adverse conditions until a more convenient time. Gottesman (25) notes that the mucoid layer functions to prevent desiccation, however, once the need is gone, *E. coli* seems to accumulate second site mutants which suppress capsule synthesis even in the absence of the Lon protease.

Polysaccharide can offer a measure of protection against phage attachment (42). This protection can be provided either by the capsule or mucoid phenotype, thus blocking the absorption to an outer membrane receptor such as the lipopolysaccharide (LPS) or to the O-antigen portion of the LPS (42). The mucoid capsule also prevents capsule polysaccharide-specific interactions such as depolymerases which can modify or lyse the outer capsule and injection of the phage nucleic acid into the bacterial cell.

The *E. coli* Rcs system.

The Gram-negative bacterium *E. coli* is capable of producing two types of capsule polysaccharide: the mucoid type I capsule polysaccharide is loosely attached and can be removed by agitation and centrifugation; the capsular type II polysaccharide is attached firmly to the outer membrane, is heat stable, and is removed by strong detergents and alkali treatment (38).

The regulation of the mucoid type I capsule polysaccharide of *E. coli* has been studied by many groups. Markovitz (44) isolated *capR (lon)* mutants of *E. coli* which overproduced a capsular polysaccharide identified as a polymer of colanic acid. He discovered that several of the enzymes necessary for synthesis of colanic acid were overproduced in *capR* mutant cells. He proposed that *capR* mutants were unable to control synthesis of colanic acid precursors uridyl-diphosphate (UDP)-galactose and UDP-glucose (45). These are the substrates for the proteins expressed by the galactose operon, in particular UDP-galactose epimerase. Markovitz (45) proposed a model of capsule polysaccharide regulation by CapR, which he believed to be a repressor. He envisioned that CapR bound to a repressor site upstream of the *gal* operon (45) thus blocking the transcription of the galactose operon.

Gottesman and coworkers introduced *lacZ* transcriptional fusions into a group of genes encoding enzymes necessary for the synthesis of colanic acid capsule (*cps::lacZ*)(69). Using second site mutagenesis of *cps::lacZ lon*$^+$ and Δ*lon* isogenic *E. coli* strains, they identified and characterized three additional genes (*rcs*: regulator of capsule synthesis)

whose products (26, 27) regulate capsule polysaccharide synthesis. Two regulators are transcriptional activators, RcsA and RcsB. A third, RcsC, is a proposed membrane bound protein which interacts with both an environmental stimulus and the *rcs*B gene product. Gottesman and coworkers proposed the following model of capsular polysaccharide regulation: RcsC is stimulated in an unknown fashion and phosphorylates RcsB. RcsB and RcsA then bind to the operator of the *cps* (capsule polysaccharide) operon and activate expression of the *cps* genes (see Figure 2). This model proposes post translational control of the RcsA gene product by the ATP-dependent Lon protease. When capsule polysaccharide is required, the Lon protease is not available to cleave RcsA; when capsule is not required, Lon cleaves RcsA and capsule synthesis is blocked. The fact that capsule synthesis is not seen in colonies incubated at 37°C but is seen at lower temperatures (32°C) suggested that the RcsA-RcsB interaction is affected by temperature (26). Gervais and Drapeau (23) identified another possible regulator, RcsF; they proposed that RcsF was the source of kinase activity and that RcsC is in fact a phosphatase (24).

Gottesman and Stout (26) analyzed the nucleotide sequences of RcsB and RcsC. These shared domains with two-component regulatory pairs (1, 56). RcsC shares homology with a set of protein kinases and RcsB has a domain in common with the targets of kinases. These targets frequently are transcriptional activators. The proposed shared homology of RcsC includes: hydrophobic domains which could be inserted into a membrane, a cytoplasmic sensor-kinase domain, and an effector-interaction domain. RcsC shares homology to the sensors CpxA, PhoR, EnvZ, and PhoM. RcsB, a soluble cytoplasmic protein, has a domain which could interact with the RcsC kinase domain as well as a C-terminal DNA binding region. RcsB shares homology with other effector proteins such as CheY, Spo0A, OmpR and NtrC (64).

E. coli RcsA also has analogs within other organisms (reviewed in 26). These are able to complement the *E. coli rcsA* mutation to promote transcription of the *cps* operon, and include two *Erwinia* analogs (4, 8, 9, 54), and one in Klebsiella (2). All share extensive homology with the C-terminal end of the predicted amino acid sequence (54). These features are shared by a

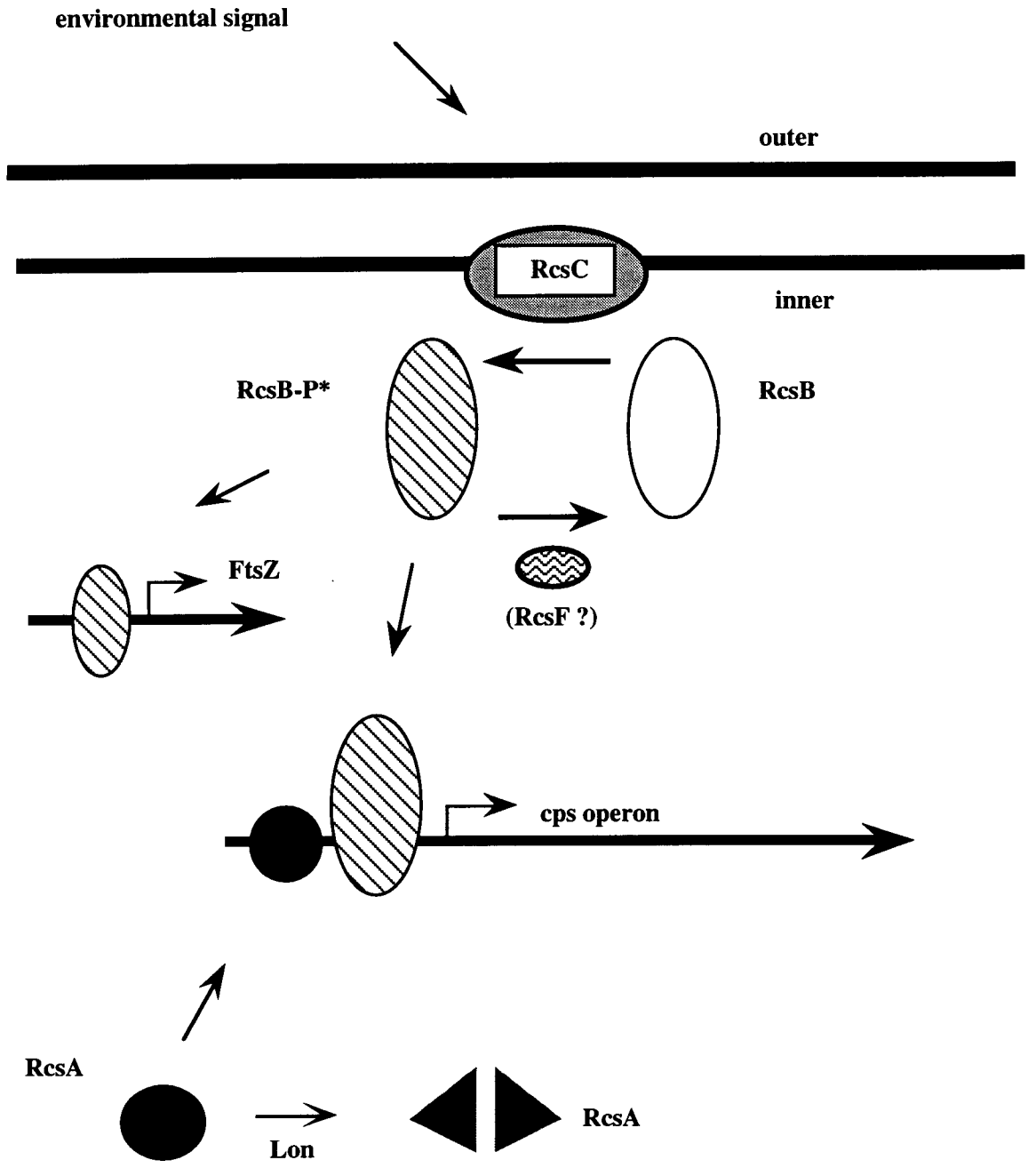

Figure 2. The model of capsule polysaccharide regulation. This figure, after Stout and Gottesman (1992), depicts RcsC as a kinase as well as a possible phosphorylase; however, an alternate model by Gervais and Drapeau (1992) suggests that RcsF is the kinase. The Lon protease regulates capsule gene expression by cleaving the transcriptional activator, RcsA. RcsB is shown to be part of both capsule and cell division (*ftsZ*)gene expression. The mechanism by which environmental signals are passed on through the outer membrane to the RcsC transmembrane protein is unknown.

family of bacterial activator proteins which include LuxR from *Vibrio fischerii* (30). In particular, computer analysis indicates a helix-turn-helix motif in the C-terminus which is proposed to be a DNA-binding domain and act as an activator of *cps* expression (54, 64)

Two component regulatory systems.

General model.

In bacteria, protein phosphorylation is one method of communication between pairs of proteins involved in responses to environmental stimuli (1). These protein pairs are part of pathways which convert environmental signals into cellular activities. This idea was first suggested by Ausubel's group which noted the similarity in domain organization among homologous regulatory pairs (51, 56).

Their model (see Figure 3) proposes that one protein of the pair is a sensor of environmental stimuli, although whether it reacts directly or indirectly to the stimuli is not known (1). The N-terminus contains domains which are predicted to be transmembrane regions with portions within the periplasmic space and which receive the environmental signal; the C-terminus receives this signal and interacts with the regulator protein by phosphorylation of the regulator, and it is this domain which shows the strongest conservation between predicted sensor proteins. The second member of this pair is the regulator which is phosphorylated and acts on transcription, either by activation or repression. The conserved domain is in the N-terminus which is proposed to receive the signal from the sensor molecule.

NtrB/NtrC.

A major enzyme used by enteric bacteria for assimilating low concentrations of NH_3 is *glnA*, glutamine synthetase. Transcription of *glnA* is from tandem promoters: *glnA*p1, which is repressed by the cytoplasmic

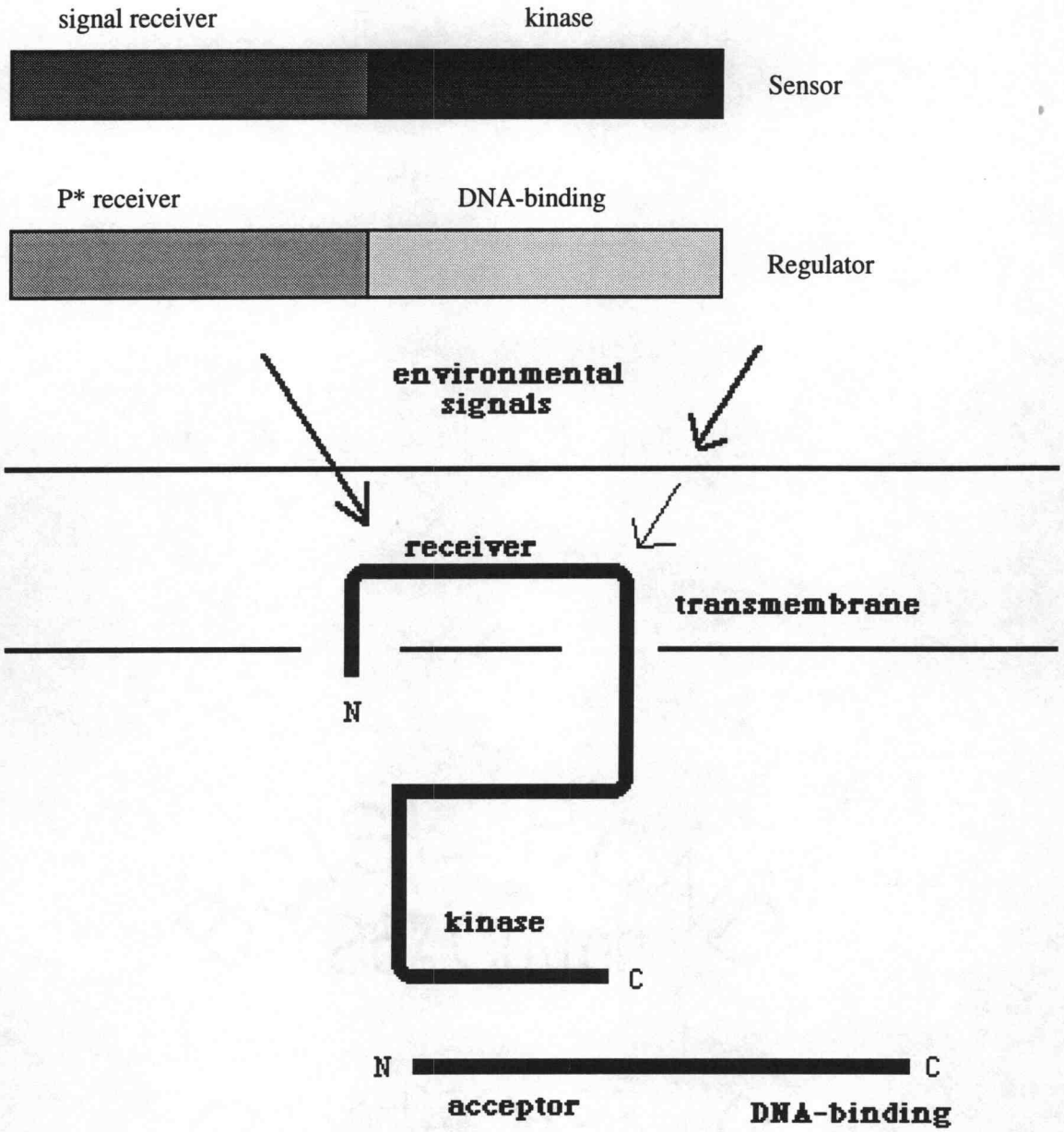

Figure 3. A generalized model of the two-component sensor-regulator pair. The N-terminus of the sensor receives, directly or indirectly, an environmental signal which is transmitted to the sensor's C-terminus. This domain interacts with the N-terminus of the regulator by phosphorylation. The activated regulator possesses regions capable of DNA-binding. The regulator acts on target gene expression by either activation or inhibition.

protein NtrC, and *glnA*p2, which is activated by NtrC (55). Activation by NtrC requires the sigma factor RpoN (σ^{54}) and phosphorylation of NtrC by the sensor NtrB. The activity of NtrB may be constitutive since phospho-NtrC is unstable and undergoes dephosphorylation by the action of the PII protein (1, 41). PII is another link in the sensory pathway and is uridylated in response to the intracellular levels of nitrogen; PII also influences the level of glutamine synthetase activity by influencing its adenylation level (41)

CheA/CheY, CheB.

Both *E. coli* and *Salmonella typhimurium* alter their movement in response to a variety of chemical and physical stimuli (53, 63). The bacteria alternate between smooth swimming and tumbling, activities stimulated by signals generated by a family of methyl-accepting transmembrane chemotaxis proteins; the response is apparent in the alteration of direction of flagellar rotation (3). In this case, the protein kinase sensor is CheA with two recipient regulators, CheY and CheB. The present model for chemotactic behavior proposes an environmental stimulus (one or more) flowing from the transmembrane proteins to CheW which stimulates CheY to undergo autophosphorylation. The transfer of the phosphate to CheY activates the clockwise rotation of the flagella. Phosphorylation of CheB, a methylesterase, promotes methylation of the methyl-accepting proteins, thus regulating their ability to pass on signals to the CheW signal coupler (5). An added level of sensitivity is provided by the phosphatase CheZ.

EnvZ/OmpC, OmpF.

The expression of the outer membrane porins OmpC and OmpF is controlled by the sensor EnvZ and the regulator OmpR (18). The transmembrane protein, EnvZ, is activated when the osmolarity of the surrounding medium increases; EnvZ is autophosphorylated and activates the cytoplasmic OmpR protein (34). In turn, OmpR activates the expression of the outer membrane protein, OmpC, and the regulator gene, *micF*. The product of *micF* is a RNA molecule which is complementary to a region of the mRNA of another outer membrane protein, OmpF, present when osmolarity levels are

low (49). The model for regulation suggests that under low osmolarity conditions the OmpF gene product is predominant; as these levels increase, the EnvZ protein is activated and phosphorylates OmpR (18). OmpR then activates the expression of the OmpC protein as well as negatively affecting the expression of OmpF (18).

PhoR/PhoA.

Bacterial alkaline phosphatase is synthesized in response to environmental phosphate levels. When inorganic phosphate concentrations are limiting, *phoA*, the structural gene for bacterial alkaline phosphatase, is expressed several hundred fold (73). *phoA* is a member of the phosphate regulon. This regulon includes several unlinked genes regulated by *psi* (phosphate starvation inducible) promoters. These are, in turn, regulated by the products of the phoBR operon. The initial model proposed that the sensor, PhoR, was activated by a signal transduced by the PstSCAB (phosphate starvation transport) transporter. PhoR then induces expression of PhoB, activating expression of *phoA*. However, there are other sensor molecules which also affect expression of PhoB. One is PhoM (CreC), which shares protein sequence similarities with other regulatory genes, is induced during growth on glucose and can induce expression of PhoB (74, 75). Another inducer of PhoB expression is an unknown sensor which is expressed in the presence of pyruvate but is PhoM independent (76). Both indicate that it is possible that there is cross regulation of the Pho regulon by a non-specific sensor in wild type cells.

VirG/VirB, VirE.

Agrobacterium tumifaciens responds to co-cultivation with plant cell suspensions by induction of *vir* (virulence) genes located on the Ti plasmid. The signal for the induction are two related phenolic compounds, acetosyringone and α-hydroxysyringone, released by wounded plant cells (62). This activates expression of vir gene products which promote the transfer of a portion of the Ti plasmid to the nucleus of the plant cells and incorporation of the bacterial DNA into the host chromosomal DNA. The regulatory model proposes that plant phenolics stimulate the VirA

transmembrane protein to activate VirG. VirG stimulates at least two genes, *virB* and *virE*, which are necessary for the bacterial DNA transfer (78).

AlgR/AlgD.

In *P. aeruginosa* infections, the invading bacteria are typically non-mucoid. The phenotype of this organism changes to mucoid, alginate-producing in patients suffering from cystic fibrosis. It is believed that the mucoid capsule is a response to localization within the host (lungs), perhaps under limiting nutrient conditions (13, 67). However, the precise environmental stimulus for the mucoid phenotype is still unknown. The alginate biosynthetic pathway absolutely depends on the activity of the *algD* gene product, GDP-mannose dehydrogenase. This enzyme catalyzes the oxidation of GDP-mannose into GDP-mannuronic acid, a precursor for alginate polymerization, and funnels the pool of sugar intermediates into alginate production (11). The activator of *algD* expression is AlgR which has been found to share strong similarities to the class of regulator proteins in two component regulatory systems. Although a kinase has not been identified which participates with AlgR, Deretic and coworkers (12) have shown it is capable of phosphorylation by the *S. typhimurium* CheA chemotaxis sensor, and this reaction is affected by the presence of small molecular weight phospho-donors such as carbamoyl phosphate and acetyl phosphate.

DegS/DegU.

In *Bacillus subtilis*, proteins not seen during vegetative growth are expressed in the postexponential stage and are apparently used to scavenge the environment for available nutrients and energy (66, 72). Among the gene products which have an effect on their synthesis are DegS and DegU. It is believed that DegS is a kinase which phosphorylates DegU in response to unknown environmental signal(s). DegU appears to be a regulatory protein with the capability of being phosphorylated and binding to DNA. At least one effect of this action is the activation of the structural gene for the extracellular protease, subtilisin, (*aprE*). Two different phenotypes are seen in *degU* or *degS* mutant cells. Cells with *degU*(Hy) or *degS*(Hy) missense mutations exhibit overproduction of several

postexponentially expressed proteins (including subtilisin, intracellular serine protease, levansucrase) and defects in flagellar synthesis, competence, and normal repression of sporulation by glucose (50). It has been proposed that these phenotypes are the result of greater phosphorylation of DegU or that the unphosphorylated form of DegU mimics the properties of the normally phosphorylated DegU. In other missense and deletion *deg*U(Hy) or *deg*S(Hy) mutants, the degradative enzyme production is deficient, but motility and competence are not affected. Here unphosphorylated DegU is an activator of competence. This suggests that the state or degree of phosphorylation of DegU directs the activation or repression of these transition state enzymes.

Crosstalk regulation.

The connection between the sensor protein and the partnered response regulator may be modified by additional inputs from sources not defined as part of the particular pathway (76). The sensor molecule is able to detect - directly from the environment, or indirectly from within the cell - stimuli which mold the response appropriate for the cell. As has been noted previously, the signal often prompts phosphorylation of the sensor which then passes on the signal by phosphorylation of the response regulator. It may be possible that the activity of the response molecule can also be regulated by other input signals from different, non-cognate, systems. Wanner (76) refers to this as cross-regulation, although there may or may not be phosphorylation involved and that the primary molecule may or may not be part of a known two-component regulatory system. While phosphorylation is a common feature of two-component systems, Wanner suggests, without example, that there may be other modes of regulation including different covalent modifications or binding of effector molecules.

The example offered for the possibility for cross-regulation is the complex nature of the Pho regulon. This regulon includes *pho*A, the gene for bacterial alkaline phosphatase as well as several other genes for the incorporation of external P sources (see Figure 4a; 76). Transport of external P_i is via the PstSCAB or Pit pathway; intracellular P_i is incorporated into ATP, the primary phosphoryl donor in metabolism. Incorporation of P_i may occur

via glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (glycolysis), phosphotransacetylase and acetate kinase (Pta-AckA) during growth on media other than acetate, succinyl-coenzyme A synthetase (tricarboxylic acid cycle), or the F_1F_0 ATP synthetase (see Figure 4b; 72). The basis for this multi-regulatory model for control of the Pho regulon may involve a regulatory link between transport of external P_i and incorporation of internal P_i into ATP. Cross regulation which involves the Pta-AckA pathway may exist to detect the ratio of ATP to acetyl phosphate, with lowered ratios causing induction. PhoM (CreC) regulation may be linked to another central pathway which detects signals for incorporation of P_i into ATP via glycolysis, the TCA cycle, or ATP synthetase. Other possible candidates for cross regulatory systems would include those which, in the absence of the cognate sensor, do not express the null phenotype, eg. sensor mutants in nitrogen control, osmoregulation, and nitrate regulation (16, 55, 57). In each of these studies, cross regulation involving the regulators NtrC, OmpR, and NarL were shown to occur in spite of the presence of mutant sensor proteins. The particular noncognate partner has not been deduced to date, and it is not known if other systems are likewise open to this type of regulation. Wanner (76) suggests that nonspecific interactions are less important than the idea of cross specificities of noncognate partners. This cross specificity adds another level of control by allowing for different stimuli to sensitize the regulator as well as a backup to low levels of the sensor due to proteolysis or inhibition of either the kinase or phosphatase specific portion of the sensor molecule.

Capsules in *Bacillus*.

There have not been extensive studies of the presence or role of capsule in *Bacillus* with the exception of the poly-D-glutamyl capsule of *Bacillus anthracis*, the pathogen responsible for anthrax. Sneath (61) notes that there have been reports of polysaccharide capsules in *B. circulans*, *B. mycoides*, *B. pumilis*, *B. alvei*; polypeptide capsule in *B. anthracis*; and polysaccharide and polypeptide containing capsule in *B. megaterium*. . Evidence of capsule polysaccharide or polypeptide has prompted research in a variety of areas.

B. anthracis.

Bacillus anthracis , the causative agent of the disease anthrax, affects sheep, cattle, horses and other types of animals (but rarely affects humans). The virulent form of the disease requires both a polypeptide capsule and a tripartite toxin; both virulence factors are plasmid encoded: the capsule on plasmid pX02, the toxin on plasmid pX01. Studies of the capsule indicate it is a poly-D-glutamyl peptide (28, 71) which is expressed by three genes necessary for capsule expression (43).

B. circulans.

A soil bacterium identified as *Bacillus circulans* was found to produce a highly viscous polysaccharide when grown aerobically in a medium containing glucose as it sole carbon source (35, 40). The polysaccharide was found to consist of D-galactose, D-mannose, L-rhamnose and D-mannuronic acid and was produced optimally at pH 7-8 in the laboratory setting. Because there was some interest in the possible use of the polysaccharide in commercial applications, special consideration was given to environmental factors which would affect viscosity and growth (35, 40). In particular, basal media with 0.1-1% glucose added provided maximal polysaccharide concentrations after incubation for 48 hr (40). Temperature had an effect on polysaccharide production, more produced at 30^{o}C and less so at either lower or higher temperatures (40). When the amount of media was altered in the 300-ml flasks used, maximal polysaccharide production occurred with 50-ml of media and aeration and production decreased with increasing amounts of media (40). In food manufacture, different sugars and organic acids are added. A variety of sugar sources have an effect on polysaccharide production; small amounts of sucrose increase viscosity more than comparable amounts of either glucose or fructose (35). Addition of organic acids decreased viscosity with increasing acid concentrations regardless of the type of acid. Polysaccharide production was affected by the change in pH (35). These reports did not investigate the regulation of polysaccharide expression.

B. licheniformis.

Bacillus licheniformis ATCC9945 produces a polypeptide capsule. Gardner and Troy (22) investigated the biosynthesis of the poly (γ-D-glutamyl) capsule after finding a polyglutamyl synthetase associated with the membrane of encapsulated *B. licheniformis.* The polymerization was specific for the L-isomer of glutamic acid and required ATP and Mg_2^{++}. The biosynthesis appeared to have parallels in the mechanism of polypeptide antibiotic synthesis with membrane associated reactions (70). The polymerization was thought to be a result of activation of L-glutamate by ATP, binding to a thioester protein, racemization to the D form, polymerization with activated glutamate-protein intermediates, then addition of this polymer with a poly (γ-D-glutamyl)-acceptor protein. McLean and coworkers (46) then found that the capsule bound multivalent aqueous ions such as Mg_2^{++}, Al_3^{++}, Mn_2^{++}, and Cu_2^{++}. They suggested a comparison with the cell wall peptidoglycan. The cell wall is held in a rigid conformation by turgor pressure and cross-linking with possible metal binding sites perhaps blocked by both orientation and cross-linking. The capsule is more loosely held with respect to the external environment and can bind more metal ions in solution. Additionally, metal ions such as Mg_2^{++} are required by cell defense systems (eg autolysins) in the cell wall, while others, such as Cr_3^{++}, are toxic. Metal binding can offer both a method for recruitment of necessary cations while preventing the localization of toxic cations away from enzymes within the membrane and even possibly in the cell wall.

Other *Bacillus* species.

Hernandez-Rodriguez and coworkers (31) compared the rheological behavior of a *Bacillus* species found in a deep oil well with that of *Klebsiella pneumonia* K8, *Arthrobacter* species, and *Xanthomonas campestris,* all of which produce copious amounts of polysaccharide. They were interested in discovering bacteria which could aid in recovery of oil trapped in water pockets within the earth which were unavailable with typical industry practices. Suitable bacteria should be capable of viscous polysaccharide production under the conditions of high temperatures,

moderate acidity, and high salt while being low in cost and pseudoplastic when pressure is applied (20). Xanthan gum provides these characteristics, but is expensive, so Hernandez-Rodriguez examined the *Bacillus* species for their ability to provide the same properties at low cost. Xanthan polysaccharide consistently demonstrated the best required properties; however the *Bacillus* polysaccharide was able to perform somewhat better than either that from *Klebsiella* or *Arthrobacter* without the apparent problem of pathogenicity.

Rationale for the experimental methods.

This review has focused primarily on the role and regulation of bacterial capsular polysaccharide production. To date, very little is known about capsule synthesis in bacilli. *Bacillus subtilis* is an ideal model system to employ in this study. *B. subtilis* genetics are well defined, and in conjunction with the high levels of capsule synthesis seen in *E. coli* cells mutant for Lon protease, the understanding of capsule regulation can be extended to other types of bacteria, such as the bacilli.

The research objectives are three-fold: 1) isolate *B. subtilis* activities which can suppress capsule synthesis in *E. coli* cells mutant for Lon protease, 2) characterize these *Bacillus* activities, and 3) examine and compare these activities with the canonical *E. coli* activities.

Materials and Methods

Strains.

Strains used in this study are listed in Table 1.

Media.

Media used in this study was LB broth except where noted. LB broth (per liter): 10 g tryptone, 5 g NaCl, 5 g yeast extract. LB-agar included 15 g/L agar added to the broth prior to autoclaving. MacConkey's agar (DIFCO, Detroit Michigan) was used in the amount of 40 g/880 ml; after autoclaving, 120 ml of sterile lactose was added and mixed. To prevent sporulation in *Bacillus* cultures, 1 ml/L sterile glucose was added after the media was autoclaved. Antibiotics added after autoclaving: ampicillin, 50 ug/ml; tetracycline, 35 ug/ml. LB-MMS plates were made by adding 0.05% methyl-methane sulfonate to cool LB agar prior to the pouring of plates.

Genomic library preparation.

Bacterial DNA was extracted by the method of Silhavey (59). Bacterial cultures (500 ml) were grown overnight at 30°C in shaking water baths. Cells were pelleted and resuspended in 5 ml of 50 mM Tris-HCl, pH 8.0, 50 mM EDTA and frozen at -20°C. 500 ul of a 10 mg/ml lysozyme in 250 mM Tris-HCl, pH 8.0 was added to frozen cell and thawed in a room temperature water bath. Just thawed cells were put on ice for 45 min. 1 ml of STEP (0.5% SDS, 50 mM Tris-HCL, pH 7.5, 400 mM EDTA, 1 mg/ml proteinase K) was added to the cells and mixed well, then heated at 50oC for 1 h with occasional, gentle mixing. Tris-buffered phenol was added (6 ml), mixed, and centrifuged for 15

Table 1. Bacterial strains used in this study.

Organism/strain	Relevant genotype	Source or Reference
Bacillus subtilis		
168	*trpC2*	BGSC[c]
Escherichia coli		
CGSC#4876(DF1071)	*gnd*-1	ECSC[d]
CGSC#5951(QE35)	Δ*gnd*	ECSC[d]
C600	F⁻ *thi*-1 *thr*-1 *leu B6 tonA21* *lacY1supE44* 1	NIH[e] strain collection
DDS90[a]	Δ*lac rcsA90::lacZ*	SG20250 + λDDS90[f]
DDS1304[a]	Δ*lac rcsA1304::lacZ*	SG20250 + λDDS1304[f]
JK5000[a]	*slpA*$_{10}$::*lacZ* immλ Kan^r	SG20250 + P1(Mu *lacG*)
JK5037[a]	*lon*$^+$*slp::lacZ*	SG20250 + P1(JK5000)
JT2181[a]	*lon*$^+$ *sulA::lacZ*	SG20250 + λ*(sfi::lacZ)*
JT2182[a]	Δ*lon sulA::lacZ*	JT4001 + λ(*sfi::lacZ*)
JT2183[a]	*lon+ rcsA::lacZ*	SG20250 + λ(SB57)
JT2184[a]	*lon*$^+$ *rcsB::lacZ*	SG20250 + λ(SB72)
JT2185[a]	Δ*lon* Δ*gal rcsA::lacZ*	JT4001 + λ(SB57)
JT2186[a]	Δ*lon* Δ*gal rcsB::lacZ*	JT4001 + λ(SB72)
JT4000[a]	Δ*lon*-510	SG1030 + P1(SG4144)
JT4001[a]	Δ*lon*-510 Δ*gal*	JT4000 + P1(SA2692)
SA2692	Δ*gal*-165	NIH[e] strain collection
SG1030[a]	F⁻ Δ*lac araD proCYA221* *zaj*-403::*Tn10*	ref. 69
SG4147[e]	*lon*$^+$ *rcsA62* lac*$^+$	N99 + lCI857 *rcsA62*[g]
SG20804[a,b]	*lon*$^+$ *rcsA rcsC* cps::lacZ*	ref. 6
SG20250[a]	Δ*lac*	ref. 27
SG20761[a,b]	*lon*$^+$ *rcsC* cps::lacZ*	ref. 6
SG20780[a,b]	Δ*lon cps::lacZ*	ref. 6
SG20781[a,b]	*lon*$^+$ *cps::lacZ*	ref. 6

Table 1. continued

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a MC4100 derived strains (Δ*lacU*169 *araD flbB rel*)

b These strains contain *cpsB*10::*lac*-Mu-immλ

c BGSC, *Bacillus* Genetic Stock Center

d ECSC, *Escherichia coli* Stock Center

e National Institutes of Health

f D. Sledjewski and S. Gottesman

g S. Gottesman strain collection

min at 1000g. The aqueous layer was transferred to a clean tube. 0.1 volume (600 ul) of 3 M NaOAc was added, mixed gently, and then 2 volumes (1.2 ml) of 95% EtOH was added. Nucleic acids which precipated were spooled out and transferred to a clean tube containing 5m of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 ug.ml RNase A, and rocked at 4°C overnight to dissolve the precipitate. An equal volume (5 ml) of $CHCl_3$ was added, inverted several times to mix, and centrigfuged at 1000g for 15 min. The aqueous layer was transferred to a clean tube, and 500 ul of 3 M NaOAc, followed by 10 ml of 95% EtOH, was added. DNA was spooled out and transferred to a clean tube and dissolved in 2 ml oof 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and stored at 4°C. The DNA concentration was determined by assessment of $OD_{260/280}$.

A library of *B. subtilis* 168 genomic DNA was created by partial *Sau3A* endonuclease digestion and insertion of DNA fragments ranging in size from 2-9 kb into the unique *BamHI* site of the multi-copy cloning vector, pBR322. This plasmid library was transformed into an *E. coli* SG20780 *Δlon cps::lacZ* strain. The resulting transformants were selected for resistance to ampicillin (50 ug/ml) on MacConkey's Lactose-ampicillin agar plates at incubation temperatures of 32°C and 39°C. White colonies indicate that *Bacillus* activities had blocked expression of the *cps::lacZ* transcriptional fusion; red colonies indicate expression had not been blocked. White colonies were assayed for their ability to survive on LB plates supplemented with methyl-methane sulfonate (MMS) (0.05% v/v). MMS resistant colonies were further studied.

β-galactosidase assays.

β-galactosidase activities were determined by the method of Miller (48). Cells were grown overnight at 32°C. 50 ul of the overnight culture was used to inoculate 5 ml of fresh media and allowed to grow to log phase (OD_{600}=0.6-0.8). One aliquot was taken for a reading at OD_{600}, another aliquot (1 ml) was placed in a test tube. To the second aliquot was added 1 drop of toluene, the contents mixed, and the tube was shaken at 30°C for 30 min. The reaction was started with the addition of O-nitrophenylgalactoside (ONPG)

(200 ul, 4 mg/ml) and the tube was agitated to mix the contents. The reaction was stopped with the addition of 500 ul of 1 M Na_2CO_3 . Optical densities (OD_{420}, OD_{550}) were recorded as well as the time interval between addition of substrate and stop solutions. Units of activity were calculated by the following formula: units = 1000 x [OD_{420} -(1.75 x OD_{550})]/ [time (min) x OD_{600} x volume (ml)].

Gluconate assay.

Gluconate metabolism was assayed on gluconate-tetrazolium plates as described by Fraenkel (17). 25.5g of Difco Antibiotic Medium #2 (Penassay agar) was mixed with 950 ml of distilled water and autoclaved. After cooling to 50°C, 50 ml of 20% sodium gluconate (final concentration, 1%) and 5 ml of 1% tetrazolium red (2,3,5 triphenyl tetrazolium chloride, final concentration, 0.005%) was added and mixed prior to plate preparation. Plates were protected from light and used within one week. Assays were performed at 37°C.

Maxicell experiments.

The maxicell experiment was performed as outlined in Silhavey (59). Cells were grown overnight in M63 media supplemented with 1% casamino acids, 0.4% glucose, 50 ug/ml ampicillin, and 0.001% thiamine-HCl. The next day, 0.2 ml of the overnight cultures were inoculated into the same medium and incubated at 32°C until the cultures reached an OD_{600} of 0.7-0.8. The cells were transferred to glass petri dishes rotating in the dark and irradiated for 17 sec to give a dose of 300 erg/mm^2. 10 ml of cells were transferred to foil-wrapped flasks and incubated for 30 min. Cycloserine was added (40 ul, 50 mg/ml stock) and the cultures were incubated overnight. The next day the cells were centrifuged for 10 min at 1500g and resuspended in M63 medium and recentrifuged. The process was repeated one more time. The cells were resuspended in 2ml of M63 medium with 0.4% glucose and incubated at 32°C for 60 min. Cells were transferred to 15 ml plastic tubes and 100 uCi/ml of ^{35}S-methionine was added. The mixture was incubated for 1 min at 32°C. Cells were centrifuged 10 min at 1500g. Pellets were resuspended in 500 ul of M63 medium, transferred to microfuge tubes and spun for 5 min. at

12,000g at 4°C. The pellets were rewashed and finally resuspended in 100 ul of sample buffer, boiled for 5 min in a water bath and stored at -20°C until use.

Separation of plasmid-encoded proteins was performed on a 12% polyacrylamide-SDS denaturing gel. Samples were reboiled for 5 min. prior to loading. Low molecular-weight prestained markers (Bio-Rad 161-0305) were loaded to determine apparent molecular weights. After the separation was completed, the gel was stained with Coomassie Brilliant Blue R-250 overnight with shaking. The next day, the gel was destained. The gel was placed in ddH_20 and shaken for 60 min, then soaked in 4% glycerol for another 60 min. The gel was then soaked in 10X gel volume (~150 ml) of Fluoro-enhance for 30 min, dried, and Kodak XAR-OMAT film was place with the gel into a film cassette with intensifying screens and exposed at -50°C. The films were developed for 2 min., washed in ddH_20 for 1 min., and fixed for another 2 min.

RESULTS

Escherichia coli Δ*lon* cells produce high levels of capsular polysaccharide (mucoid phenotype) and are sensitive (induced lethal phenotype) to DNA-damaging agents such as ultraviolet light, methyl-methane sulfonate and mitomycin C. *E. coli* Δ*lon* cells are well suited for identifying those activities that decrease capsule synthesis. *Bacillus subtilis* activities were selected for their ability to suppress the mucoid phenotype of *E. coli* Δ*lon* cells. Wildtype *B. subtilis* 168 genomic DNA, partially digested by the restriction endonuclease *Sau3A*, was cloned into the multicopy plasmid vector, pBR322. This plasmid library was transformed into *E. coli* Δ*lon* cells (SG20780), which carry a *lacZ* transcriptional fusion to the chromosomal copy of the capsule genes (*cps::lacZ*). In this approach, expression of the capsular genes was assayed as a function of β-galactosidase activity.

When SG20780 Δ*lon cps::lacZ* cells are plated on MacConkey's Lactose agar, colonies appeared red since Lon protease was not present to degrade the capsule gene transcriptional activator, RcsA (regulator of capsule synthesis). In contrast, wildtype *E. coli* with a *cps::lacZ* fusion (SG20781) are Lac^+ (white) on MacConkey's Lactose agar, due to the availablility of Lon protease, which degrades its substrate, RcsA. If a *B. subtilis* activity from the plasmid library was able to suppress capsule synthesis in SG20780, then these *E. coli* Δ*lon* cells appeared white on MacConkey's Lactose agar.

After transformation of the *B. subtilis* plasmid library into SG20780, selection for a capsule suppressing activity was performed at both 32°C and 39°C, because some activities may be produced in quantities that are toxic to the cell at one temperature but not at the other temperature, and because capsular polysaccharide is not produced at higher temperatures, whereas it is overproduced at lower temperatures. 30,000 ampicillin resistant transformants were examined for the Lac^- phenotype on MacConkey's Lactose agar containing ampicillin. Lac^- ampicillin resistant transformants were detected at a frequency of 0.1%.

Those transformants that were Lac$^-$ (white) were screened for resistance to the DNA damaging agent methyl-methane sulfonate (MMS), an inducer of the *E. coli* SOS (DNA damage) response. One of the proteins induced during the SOS response is an inhibitor of cell division (SulA). Wildtype *E. coli* (SG20781), grown in the presence of MMS, produces short filaments that resolve into individual cells after the source of damage is removed. Resolution of the cells depends on the action of the Lon protease, which degrades SulA so that cell division can proceed. In the absence of Lon, filamentation remains unresolved and the cells die. Two ampicillin resistant Lac$^-$ transformants of SG20780 were found which were MMS resistant and failed to produce filaments. The results of these screenings are displayed in Table 2. Recombinant plasmids conferring the capsule suppressing, MMS resistant phenotype were named pJET32.2 (isolated at 32^{o}C) and pJET39.20 (isolated at 39^{o}C). To verify that the activity of interest was carried by the plasmid, pJET32.2 and pJET39.20 were isolated from these transformants and retransformed into SG20780 (*E. coli* Δ*lon*) cells. Both plasmids conferred to recipient cells the Lac$^-$ phenotype on MacConkey's Lactose ampicillin agar upon retransformation, and this phenotype was stably reproducible upon incubation at a variety of temperatures. Transformants were equally resistant, as compared to their wildtype counterpart, to such DNA damaging agents as UV irradiation, mitomycin C or MMS. Because SG20780 contains a *cps::lacZ* chromosomal fusion, these *E. coli* Δ*lon* cells are not phenotypically mucoid although capsule synthesis is high as reported by the *lacZ* fusion. An *E. coli* Δ*lon* strain lacking the *cps::lacZ* fusion (JT4000), and phenotypically mucoid, was used to determine if pJET32.2 and pJET39.20 actually suppressed capsule synthesis. This proved to be the case, as the mucoid phenotype was not detected on LB ampicillin agar plates containing JT4000 cells transformed with either pJET32.2 or pJET39.20.

SG20781 wildtype *lon*$^+$ *E. coli* cells are Lac$^-$ on MacConkey's Lactose agar and are resistant to MMS. β-galactosidase activity, a measure of *cps::lacZ* activity, is negligible in SG20781 containing either the pBR322 vector alone or pBR322 plus *Bacillus* DNA inserts (pJET32.2 or pJET39.20) (Table 2). In SG20780 *E. coli* Δ*lon* cells, the presence of the pBR322 vector alone did not affect the *lon*$^-$ phenotypes of mucoidy and sensitivity to MMS. However, in the presence of either pJET32.2 or pJET39.20, the colonies

TABLE 2. Multicopy *B. subtilis* activities suppress *E. coli* Δ*lon* phenotype.

Relevant genotype[a]	Plasmid[b]	Phenotype		
		MMS[c]	Lac[d]	β-galactosidase activity[e]
lon$^+$ *cps::lacZ*	pBR322	R	-	0.3
Δ*lon cps::lacZ*	pBR322	S	+	221.9
Δ*lon cps::lacZ*	pJET32.2	R	-	5.1
Δ*lon cps::lacZ*	pJET39.20	R	-	17.6
lon$^+$ *cps::lacZ*	pJET32.2	R	-	0.1
lon$^+$ *cps::lacZ*	pJET39.20	R	-	0.2

a *E. coli* MC4100 derivatives; contains Δ*lacU169 araD flbB rel cpsB10::lac*-Mu-*imm* 1. Strain designation: *E. coli lon*$^+$*cps::lacZ*: SG20781; *E. coli* Δ*lon cps::lacZ*: SG20780.

b Chromosomal DNA was isolated from *B. subtilis* 168 (wildtype), partially digested with *Sau3A* enzyme, and cloned into pBR322. pJET32.2 and pJET39.20 were selected from this plasmid library for their ability to suppress both of the desired Δ*lon* phenotypes.

c MMS phenotype was determined on LB agar containing 0.05% methylmethane sulfonate (MMS). Cell growth was evaluated at 30°C, 32°C, 37°C, and 42°C. R = Resistant; S = Sensitive

d Lac phenotype was determined on lactose-MacConkey indicator agar containing ampicillin (50 mg/ml) (MacLacAmp) at 30°C, 32°C, 37°C, and 42°C. -, Lac$^-$ (white on MacLacAmp agar); +, Lac$^+$ (red on MacLacAmp agar).

e Cells were grown in LB with ampicillin (50 mg/ml) at 32°C and assayed for β-galactosidase. Specific activity expressed in Miller units (1972). The values represent the mean of three assays.

were white on MacConkey's Lactose agar, resistant to MMS, and β-galactosidase activity was reduced 43- and 17-fold, respectively, when compared to the vector control values(Table 2). Although pJET32.2 and pJET 39.20 were selected at different temperatures, incubation temperature did not affect either the Lac phenotype or resistance to MMS.

The similarities between the effects of pJET32.2 and pJET39.20 suggested that each contained the same insert of *B. subtilis* DNA. Figure 5 shows the result of restriction endonuclease digests analysis of the two plasmids. pJET32.2 contains a 5.5 kb fragment from *B. subtilis* while pJET39.29 contains a 4.5 kb insert. There are no overlapping restriction sites between the two plasmid inserts.

The phenotype conferred by the *B. subtilis* activities resembles that of an *E. coli* strain with a functional Lon protease. The *E. coli* Lon protease is an ATP-dependent serine protease that contains distinct and non-overlapping domains identified as a serine catalytic site, an ATP-binding site and possible DNA-binding sites (7). The serine catalytic site lies within an 800 bp *XmnI* DNA fragment of the *E. coli lon* open reading frame (ORF). This fragment was chosen because of its distinctive functional nature, and a labeled DNA probe was created to determine whether it would bind to pJET32.2 or pJET39.20 DNA sequences or to any portion of the *B. subtilis* genome. DNA from the two plasmids, as well as genomic *B. subtilis* DNA, was digested with *HindIII*, separated on an agarose gel, blotted to nitrocellulose and probed with the *XmnI* DNA *lon* fragment from *E. coli*. The probe did not hybridize to either plasmid insert or to the genomic *B. subtilis* DNA (data not shown).

Although the Southern blot analysis did not reveal similarities at the DNA level, the possibility remained that this negative result was due to differences in codon usage rather than in amino acid composition. If the two plasmid activities resembled the *E. coli* Lon protease in function, then amino acid composition and structure might be similar, and an antibody to Lon protease would be expected to bind to a homologous protein from whole cell extracts of *E. coli* Δ*lon* cells containing either of the *B. subtilis* activities. Polyclonal antibody to *E. coli* Lon protease (courtesy of S. Gottesman) was used

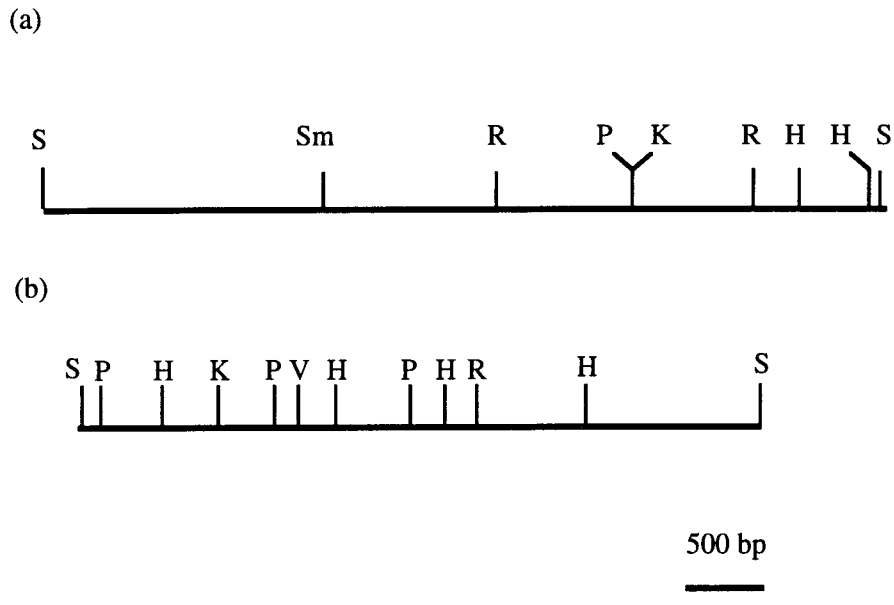

Figure 5. Restriction maps of pJET32.2 and 39.20. *Bacillus subtilis* genomic DNA partially digested with Sau3A endonuclease and ligated to the multicopy plasmid vector, pBR322, digested with BamHI. The inserts, (a) pJET32.2 and (b) pJET39.20 are oriented with the Sau3A site nearest to the pBR322 tetracycline promoter on the left. Abbreviations: A, AvaI; H, HindIII; K, KpnI; P, PstI; R, EcoRV; S, Sau3A; V, PvuII.

to probe a blot of whole cell extracts (separated on a SDS-polyacrylamide gel) from *E. coli* Δ*lon* cells containing pJET32.2 and pJET39.20 as well as from crude whole cell extract from wildtype *B. subtilis* cells. The antibody failed to detect a Lon like protein in *E. coli* Δ*lon* cells with either pJET32.2 or pJET39.20; however, it did react with a protein in the *B. subtilis* extract which has a molecular weight similar to that of the *E. coli* Lon protease (K.P. Dierksen, unpublished results).

Structurally, there were no common features at the DNA or protein level between Lon protease and the activities associated with pJET32.2 or pJET39.20. However, activities contained on *B. subtilis* inserts in pJET32.2 and pJET39.20 gave rise to phenotypes which mimic that of the *E. coli* Lon protease: absence of capsule polysaccharide synthesis and resistance to DNA-damaging agents. It was therefore possible that these *B. subtilis* activities restored the *lon*$^+$ phenotype because expression of the Lon substrates was affected, rather than proteolysis of either RcsA or SulA. By using *E. coli* strains containing either *sulA*::*lacZ* or *rcsA*::*lacZ* transcriptional fusions, and assaying for β-galactosidase activities, the effect of either plasmid containing *B. subtilis* activities could be determined. Table 3 lists the activities of the *sulA*::*lacZ* and *rcsA*::*lacZ* transcriptional fusions in the presence of the *Bacillus* inserts. In the case of the Lon substrate, the cell division inhibitor, SulA, there was not a significant difference in *sulA::lacZ* expression in *E. coli* *lon*$^+$ or Δ*lon* cells containing the vector pBR322. When pJET32.2 is present, expression of the *sulA*::*lacZ* transcriptional fusion is reduced by only 1.4 fold, as compared to the pBR322 vector control, when assayed in *lon*$^+$ cells. A similar reduction (1.8 fold) was detected when these constructs were assayed in Δ*lon* cells. pJET39.20 in *lon*$^+$ cells reduced expression of *sulA::lacZ* by only 2 fold. In Δ*lon* cells, the activity of the same plasmid did not affect expression of *sulA::lacZ*. Based on these results, *sulA::lacZ* expression appeared not to be dramatically affected by the presence of either *B. subtilis* DNA sequence. There was not a significant difference in *rcsA::lacZ* expression between *E. coli* *Lon*$^+$ or Δ*lon* cells containing the vector pBR322. When assayed in *lon*$^+$ cells, pJET32.2 and pJET39.20 both increased expression of the *rcsA::lacZ* transcriptional fusion, 2.8 and 1.6 fold, respectively. In Δ*lon* cells, the activity of pJET32.2 did not significantly affect expression of *rcsA::lacZ* .

TABLE 3. Effect of pJET32.2 and pJET39.20 on synthesis of the *E. coli* Lon substrates, SulA and RcsA.

Relevant genotype[b]	Plasmid	β-galactosidase activity[a]	
		sulA::lacZ	*rcsA::lacZ*
lon$^+$	pBR322	91.8	128.3
lon$^+$	pJET32.2	63.6	362.6
lon$^+$	pJET39.20	45.8	211.3
Δ*lon*	pBR322	81.9	136.7
Δ*lon*	pJET32.2	44.7	166.1
Δ*lon*	pJET39.20	77.8	51.0

a Cells were grown in LB with ampicillin (50 mg/ml) at 32°C and assayed for β-galactosidase. Specific activity expressed in Miller units (1972). The values represent the mean of three assays.

b Isogenic sets of *lon*$^+$ and Δ*lon E. coli* strains containing either *sulA::lacZ* or *rcsA::lacZ* transcriptional fusion located chromosomally at the *E. coli* λ *att* site were used. Strain designation (MC4100 derivatives): *E. coli lon*$^+$*sulA::lacZ*: JT2181; *E. coli* Δ*lon sulA::lacZ*: JT2182; *E. coli lon*$^+$*rcsA::lacZ*: JT2183; *E. coli* Δ*lon rcsA::lacZ*: JT2184.

pJET39.20 decreased expression of the *rcsA::lacZ* transcriptional fusion in Δ*lon* cells by 2.7 fold. Based on these results, it is clear *rcsA::lacZ* expression does not dramatically change in the presence of either *B. subtilis* DNA sequence.

Trempy and Gottesman (68) described the multicopy phenotype of *E. coli* Alp (activator of Lon-like protease), which complements the *E. coli* Δ *lon* phenotype of mucoidy and sensitivity to DNA damaging agents. A multicopy plasmid carrying Alp activity (pAlp) restored *E. coli* Δ*lon* to a lon^+ phenotype through activation of the Slp integrase (Trempy and Gottesman, submitted). The model proposed to explain restoration of a lon^+ phenotype by Alp and Slp suggests that Alp activation of Slp gave rise to an excision event which results in the expression of a protease, the substrate specificity of which overlaps with that of Lon. It seemed reasonable to ask if an activity from either pJET39.20 or pJET32.2 exhibited an affect on Slp expression similar to that exerted by Alp. Table 4 shows that only the presence of pAlp resulted in high level expression of the *slp::lacZ* transcriptional fusion; the presence of either the vector, pJET32.2 or pJET39.20, resulted in low level expression of the *slp::lacZ* transcriptional fusion.

The activity of interest was located in each plasmid by deletion of parts of the cloned *B. subtilis* inserts and subsequent reassessment of the resulting phenotype on MacConkey's Lactose agar (W Ebel, unpublished results). pJET32.2 was partially digested with *EcoRV*, and the 5'-3.0 kb fragment, along with the vector fragment from the unique vector *EcoRV* site to the *BamHI* cloning site, was removed, and the resulting 6.5 kb fragment was religated. When this construct was transformed into Δ*lon cps::lacZ* cells, the transformants were red, indicating that the deletion consruct did not complement the lon^- phenotype on MacConkey's Lactose ampicillin agar. pJET39.20 was similarly treated and the 5'-2.6 kb fragment, along with the vector from the cloning site to the unique *EcoRV* site was removed, and the 6.2 kb fragment was religated. This deletion construct did not complement the lon^- phenotype on MacConkey's Lactose agar.

The plasmid inserts were subcloned into the phage sequencing vectors, M13mp18 and M13mp19 and the plasmid inserts sequenced (W. Ebel and J.E. Trempy, unpublished results). The DNA insert carried by pJET32.2 has

TABLE 4. Effect of pJET32.2 and pJET39.20 on expression of the Slp integrase.

Relevent genotype[b]	Plasmid	β-galactosidase activity[a] *slp::lacZ*
lon$^+$	pBR322	38.2
lon$^+$	pAlp	575.2
lon$^+$	pJET32.2	32.6
lon$^+$	pJET39.20	25.3

[a] Cells were grown in LB with ampicillin (50 mg/ml) at 32°C and assayed for β-galactosidase. Specific activity expressed in Miller units (1972). The values represent the mean of three assays.

[b] Strain designation (MC4100 derivative): *E. coli lon*$^+$*slp::lacZ*: JK5037

homology to the *B. subtilis gnt* operon which codes for enzymes needed in gluconate metabolism. The *gnt* operon is oriented towards the promoter for tetracycline resistance in pBR322. The DNA insert carried by pJET39.20 has homology to ORF5 (open reading frame 5) which lies upstream of the *B. subtilis pdh* operon (pyruvate dehydrogenase). The function of ORF5 is unknown, and is unrelated to the function ascribed to pyruvate dehydrogenase activity. The orientation of ORF5 is also toward the promoter of the tetracycline resistance gene in pBR322.

Since the activities from pJET32.2 represents enzymes involved in gluconate metabolism, it seemed reasonable to ask if pJET32.2 could complement an *E. coli* mutant deficient in gluconate metabolism (*gnd*$^-$). pJET32.2 was transformed into *E. coli gnd* strains (CGSC#4876: *gnd*$^-$*1*; CGSC#5951: Δ*gnd*) and screened on gluconate-tetrazolium plates. Metabolism of gluconate would result in colonies which appear red on this medium; otherwise they would appear white. Either strain harboring pJET32.2 produced only white colonies (Table 5), indicating that pJET32.2 did not complement the *gnd*$^-$ phenotype.

There appeared to be a slight decrease in RcsA expression when *rcsA::lacZ* activity was examined in *E. coli* Δ*lon* cells containing pJET39.20 (Table 3). This result was examined further in other *E. coli* strains which contain different *rcsA::lacZ* transcriptional fusions. The *rcsA::lacZ* transcriptional fusion in strain DDS90 was expressed at low levels whereas the *rcsA::lacZ* transcriptional fusion in strain DDS1304 was expressed at high levels. The difference in *rcsA* expression in these two strains reflected the amount of the *rcsA* regulatory region fused to a promotorless *lacZ*. As seen in Table 6, pJET39.20 does not have any effect on expression, and the earlier observation of an initial decrease appears to have been an anomalous result. In addition to RcsA, a second transcriptional activator, RcsB, regulates capsule gene expression. Excess RcsB results in a mucoid phenotype in the absence of RcsA, suggesting that the role of RcsB in activating capsule gene expression is far more significant than that of RcsA (26). In contrast, excess RcsA does not give rise to a mucoid phenotype in the absence of RcsB, demonstrating that capsule gene expression is absolutely dependent on RcsB and cannot be bypassed by excess RcsA (64). Furthermore, Gottesman and Stout

TABLE 5. Complementation test for gluconate metabolism.

Relevant genotype[a]	Plasmid	Color[b]
gnd+	pBR322	red
gnd-	pBR322	white
gnd-	pJET32.2	white
Δ*gnd*	pBR322	white
Δ*gnd*	pJET32.2	white

a Strain designation: *E. coli gnd*+: SG20781; *E. coli gnd-1*: DF1071; *E. coli* Δ*gnd*: QE75.

b Cells were grown on gluconate tetrazolium agar plates containing ampicillin (50 mg/ml)at 37°C and scored for color (Frankel, 1968). Red: gluconate metabolized; White: gluconate not metabolized.

demonstrated that the stability of RcsA is enhanced in the presence of excess RcsB, suggesting that RcsB protects RcsA from Lon directed degradation (64). In light of these observations, one would predict that, if RcsB expression was to be repressed by an activity encoded on pJET39.20, then expression of capsule polysaccharide would be reduced. pJET39.20 does not affect the expression of a *rcsB::lacZ* transcriptional fusion in either a *lon*$^+$ or Δ*lon* *E. coli* strain (Table 7), indicating that the requirement for RcsB in capsule synthesis is met.

Another requirement for the expression of capsule genes is the appropriate interaction of RcsB with a membrane sensor kinase, RcsC. Interference with the transfer of signal from the membrane bound RcsC to the transcriptional activator, RcsB, results in the repression of capsule gene expression. It remained to be determined if pJET39.20 repressed capsule synthesis by affecting the interaction of RcsC with RcsB. An *E. coli lon*$^+$ strain containing a mutation in *rcsC (rcsC137)* produces capsule to a higher extent than an *E. coli* Δ*lon* strain (26; Table 8). Presumably this mutation gives rise to a constitutively active sensor-kinase that transfers signal to RcsB, leading to RcsB activation, regardless of growth conditions (26). If an activity from pJET39.20 was interfering with the interaction of RcsC with RcsB, then the prediction would be that the *E. coli lon*$^+$ *rcsC137* strain would not express capsule if that strain contained pJET39.20. Table 8 displays these results. *cps::lacZ* expression is high in the *E. coli lon*$^+$ *rcsC137 cps::lacZ* strain when it contains the vector, pBR322. No significant difference in *cps::lacZ* expression was detected when this *E. coli* strain contains pJET39.20, indicating that the interaction between RcsC and RcsB has not changed. To extend this analysis further, The effect of pJET39.20 on the interaction of RcsB with RcsC in the absence of RcsA was evaluated. Table 9 displays these results. *cps::lacZ* expression is low in the *E. coli lon*$^+$ *rcsA*$^-$ *rcsC137 cps;;lacZ* strain when it contains the vector, pBR322. No significant difference in *cps::lacZ* expression was detected when this *E. coli* strain contained pJET39.20.

An additional requirement for the expression of capsule genes is stabilization of the transcriptional activator, RcsA, so that it may interact in concert with RcsB at the cps promoter. One way that RcsA can be stabilized is through the inactivation of Lon protease (25). Another way RcsA can be

TABLE 6. Effect of pJET39.20 on the expression of the capsule gene activator, RcsA.

Relevant genotype[b]	Plasmid	β-galactosidase activity[a]
rcsA90::lacZ	pBR322	5.52
rcsA90::lacZ	pJET39.20	10.25
rcsA1304::lacZ	pBR322	160.19
rcsA1304::lacZ	pJET39.20	164.39

a Cells were grown in LB with ampicillin (50 mg/ml) at 32°C and assayed for β-galactosidase. Specific activity expressed in Miller units (1972). The values represent the mean of three assays.

b Strain designation (MC4100 derivative): *E. coli lon*$^+$*rcsA90::lacZ*: DDS90; *E. coli lon*$^+$*rcsA1304::lacZ*: DDS1304.

TABLE 7. Effect of pJET39.20 on the expression of the capsule gene activator, RcsB.

Relevant genotype[b]	Plasmid	β-galactosidase activity[a] *rcsB::lacZ*
lon$^+$	pBR322	151.3
lon$^+$	pJET39.20	123.7
Δ*lon*	pBR322	192.9
Δ*lon*	pJET39.20	191.7

a Cells were grown in LB with ampicillin (50 mg/ml) at 32°C and assayed for β-galactosidase. Specific activity expressed in Miller units (1972). The values represent the mean of three assays.

b Isogenic sets of *lon*$^+$ and Δ*lon* *E. coli* strains containing *rcsB::lacZ* transcriptional fusion located chromosomally at the *E. coli* λ *att* site were used. Strain designation (MC4100 derivatives): *E. coli lon*$^+$*rcsB::lacZ*: JT2184; *E. coli* Δ*lon rcsB::lacZ*: JT2186.

stabilized is by mutating *rcsA* and then screening mutants for RcsA proteins that are resistant to Lon degradation (25). A mucoid *E. coli lon*$^+$ strain was identified which contains a mutation in *rcsA* (*rcsA62*) that results in the expression of a RcsA protein resistant to Lon degradation (25). If an activity from pJET39.20 interfered with the interaction of RcsA with RcsB or interfered with the interaction of RcsA with the *cps* promoter, then the prediction would be that the *E. coli lon*$^+$ *rcsA62* strain would not express capsule if this strain contained pJET39.20. Table 10 displays these results. pJET39.20 suppressed expression of the mucoid phenotype in this *E. coli* strain.

Proteins encoded by the *B. subtilis* inserts of pJET32.2 and pJET39.20 were identified by the maxicell procedure (51). This procedure reduces chromosomal protein expression relative to plasmid borne activities. Figure 6 shows the result of this experiment. pJET32.2 expressed a large protein band of about 45-49 kDa. Examination of either DNA strand of the *B. subtilis gnt* operon reveals that only the open reading frame for *gntP* (permease) was large enough to encode a protein of this size. pJET39.20 expressed a protein band not detected in the control lanes, and this protein appeared to be approximately 31 kDa. The predicted weight of ORF5 is 26 kDa. The difference between the predicted weight (26 kDa) and that determined on the gel (31 kDa) may reflect problems associated with alignment with the molecular weight markers or differences in migration between denatured and native proteins.

TABLE 8. Effect of pJET39.20 on the interaction of RcsB with RcsC as measured by *cps::lacZ* expression.

Relevant genotype[b]	Plasmid	β-galactosidase activity[a] *cps::lacZ*
lon$^+$	pBR322	0.3
Δ*lon*	pBR322	39.0
lon$^+$ *rcsC137*	pBR322	353.6
lon$^+$ *rcsC137*	pJET39.20	440.4

[a] Cells were grown in LB with ampicillin (50 mg/ml) at 32°C and assayed for β-galactosidase. Specific activity expressed in Miller units (1972). The values represent the mean of three assays.

[b] Strain designation (MC4100 derivatives): *E. coli lon*$^+$*cps::lacZ*: SG20781; *E. coli* Δ*lon cps::lacZ*: SG20780; *E. coli lon*$^+$*rcsC137 cps::lacZ* SG20761.

TABLE 9. Effect of pJET39.20 on the interaction of RcsB with RcsC in the absence of RcsA and as measured by *cps::lacZ* expression.

Relevant genotype[b]	Plasmid	β-galactosidase activity[a] *cps::lacZ*
lon$^+$	pBR322	0.8
Δ*lon*	pBR322	80.9
lon$^+$*rcsA*$^-$*rcsC137*	pBR322	15.1
lon$^+$*rcsA*$^-$*rcsC137*	pJET39.20	27.1

[a] Cells were grown in LB with ampicillin (50 mg/ml) at 32°C and assayed for β-galactosidase. Specific activity expressed in Miller units (1972). The values represent the mean of three assays.

[b] Strain designation (MC4100 derivatives): *E. coli lon*$^+$*cps::lacZ*: SG20781; *E. coli* Δ*lon cps::lacZ*: SG20780; *E. coli lon*$^+$*rcsA*$^-$ *rcsC137 cps::lacZ*: SG20804.

TABLE 10. Effect of pJET39.20 on an *E. coli* *lon*$^+$ strain that overproduces capsule.

Relevant genotype[a]	Plasmid	Phenotype[b]
lon$^+$*rcsA62*	-	Mucoid
lon$^+$*rcsA62*	pBR322	Mucoid
lon$^+$*rcsA62*	pJET39.20	Nonmucoid

[a] Strain designation (N99 derivative): *E. coli* *lon*$^+$*rcsA62*: SG4147.

[b] Phenotype was evaluated on MacConkey's Lactose agar plates at 32°C. Ampicillin (50 mg/ml) was included when appropriate.

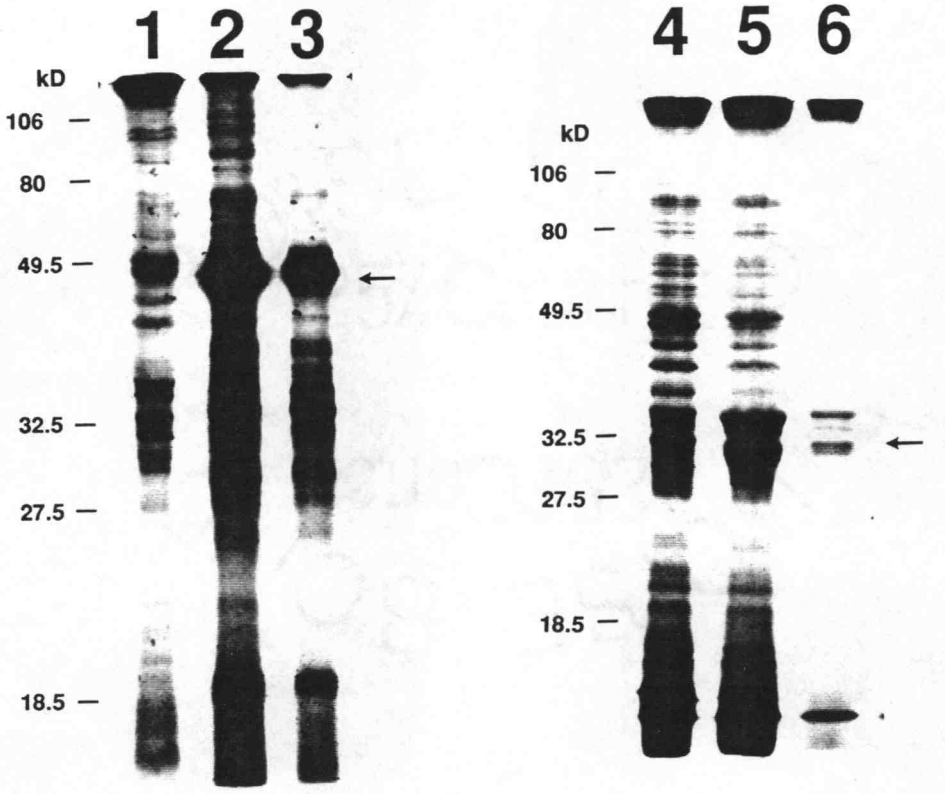

Figure 6. Maxicell plasmid protein profiles. The expressed proteins from pJET32.2 and pJET39.20, prepared as in Silhavey (1984), separated on a 12% SDS-polyacrylamide gel. Lane: 1, SG20780 + pBR322; 2, SG20780 + pJET32.2 (undiluted); 3, SG20780 + pJET32.2 (diluted 1:10); 4, SG20780 + pBR322; 5, SG20780 + pJET39.20 (undiluted); 3, SG20780 + pJET39.20 (diluted 1:10). Prestained molecular weight markers used to size the proteins are indicated to the side.

DISCUSSION AND SUMMARY

The rationale for this project was to identify activities from *Bacillus subtilis* which could suppress the mucoid phenotype in *Escherichia coli*. Laboratory strains do not exhibit the mucoid phenotype under optimal growth conditions. One way to induce the mucoid phenotype in the laboratory is to inactivate Lon protease. Thus, an *E. coli* strain deleted for *lon* is an ideal system to identify those mechanisms that repress expression of the mucoid phenotype. Nothing had been known about the expression of polysaccharide in the bacilli, although there had been numerous reports that a mucoid phenotype occurs in growth conditions outside of the laboratory, and this phenotype is of potential significance to a variety of industries. Given the accessibility of a mucoid *E. coli* strain, a prediction was made that there were activities from a *B. subtilis* chromosomal plasmid library which might suppress the mucoid phenotype in this *E. coli* strain. Two classes of *B. subtilis* activities were identified by this selection method. Class I represented activities that suppressed only the mucoid phenotype, while Class II represented activities that suppressed the mucoid phenotype in addition to another phenotype associated with loss of Lon protease activity - sensitivity to DNA damaging agents. A decision was made to focus on Class II activities because only two unique *B. subtilis* DNA fragments were identified that conferred the desired phenotype in *E. coli*.

Six possible interpretations for the suppression of mucoidy and sensitivity to DNA damaging agents by the *B. subtilis* activities were considered: 1) proteolysis - the *E. coli* Lon substrates are also substrates for a *B. subtilis* protease, and this protease is either similar to Lon or overlaps it in substrate specificity; 2) repression - expression of the *E. coli* Lon substrates, RcsA and SulA, are repressed by the *B. subtilis* activities, thus nullifying the effects of the *lon* deletion; 3) activation - the *B. subtilis* activities are positive regulators able to correctly activate, directly or indirectly, the expression of alternative protease genes in *E. coli*; 4) interference - the *B. subtilis* activities encode proteins that interfere with specific protein-protein interactions necessary in both the capsule and SOS (DNA damage) responses;

5) titration - the *B. subtilis* activities represent DNA sequences which, when present in multicopy, titrate away repressors from *E. coli* protease genes, thereby promoting expression of otherwise "silent" gene functions; or 6) modification - the *B. subtilis* activities mark the Lon substrates for proteolysis, and this modification is interpreted by alternative *E. coli* proteolytic mechanisms.

The most obvious interpretation was that a Lon-like protease had been identified in *B. subtilis* and that this protease was able to substitute, in function, for the *E. coli* Lon protease. Structural analysis revealed that the *B. subtilis* inserts of pJET32.2 and pJET39.20 do not hybridize to an *E. coli lon* DNA probe, and Lon antibody fails to react with proteins expressed by the DNA inserts. This result does not eliminate the possibility of a *B. subtilis* proteolytic mechanism which restores *E. coli* Δ*lon* cells to a *lon*$^+$ phenotype. However, immunoblot analysis of total protein from a crude *B. subtilis* extract with Lon antibody revealed the presence of a Lon-like protein. If a Lon-like activity exists in *B. subtilis*, the putative *B. subtilis lon* DNA ought to have been recovered in this selection. One possible reason why such an activity was not detected is that the *B. subtilis lon* gene is lethal to *E. coli* in multicopy; multicopy portions of the *E. coli lon* gene may be propagated in *E. coli*, however, multicopy propagation of the full length gene is lethal to the cells (JE Trempy, unpublished results).

Neither *B. subtilis* activity achieved its effect by repressing expression of the Lon substrates, RcsA or SulA, which might have also led to the same *lon*$^+$ phenotype. Neither of the *B. subtilis* activities functions in a manner similar to the *E. coli* Alp. The Alp activity complements the *lon* phenotype by activating the expression of Slp integrase, which is responsible for a recombination event leading to protease expression.

Of the three possibilities not dismissed by the above considerations, those which propose that the *B. subtilis* activities interfere with necessary protein-protein interactions seem the most plausible. DNA sequence analysis and identification of the proteins expressed from these two DNA sequences revealed that the permease (GntP) encoded by the gluconate operon was expressed from the *B. subtilis* insert of pJET32.2, and a protein of

the approximate size of that predicted for ORF5, which lies upstream of the pyruvate dehydrogenase operon, was expressed from the *B. subtilis* insert of pJET39.20.

The *B. subtilis* GntP protein appears to be a membrane protein which allows gluconate to enter the cell. How could this permease affect *E. coli* Δ*lon* hosts so that the mucoid phenotype is suppressed or *cps::lacZ* expression was turned off or sensitivity to DNA damaging agents was lifted?

There are, in general, three methods which the bacilli can use to bring solutes within the cell. These transport systems may involve the direct activation by ATP hydrolysis, phosphoryl transfer from PEP (phosphoenol pyruvate) to a sugar substrate during group translocation, or facilitated transfer driven by a chemiosmotic ion gradient (58). *B. subtilis* acquires gluconate by a facilitated transfer (solute:cation symport) of the sugar along with a cation (H^+or Na^+). The influx of gluconate is determined by the gluconate (*gnt*) operon which encodes four proteins (GntRKPZ) (19). The operon is repressed by glucose, suggesting that it is subject to catabolite repression, and induced by the presence of gluconate. The GntP protein is the permease which permits the influx of gluconate, along with a cation, against a concentration gradient that is driven by a negative potential with respect to the extracellular environment.

The *B. subtilis* Gnt permease is expressed in multicopy from pJET32.2. What would be the effect of this overexpression of a gram-positive protein within a gram-negative cell? Assuming it is expressed correctly, the permease could be inserted into the membrane in addition to the *E. coli* permease or it could form heterologous complexes with either the cognate and/or other permeases or it could prove incapable of insertion into the membrane. If it did not insert into the membrane, one would not expect that lactose metabolism, measured by the *cps::lacZ* transcriptional fusion, would be affected. If it provided additional avenues for gluconate influx, lactose metabolism ought not to be affected. However, if heterologous complexes occured with other permeases, and if these proteins no longer were capable of proper function, it would explain both the absence of *cps::lacZ* expression as well as the apparent insensitivity to methyl-methane sulfonate (9). If

these heterologous proteins were capable of forming correctly functioning permeases, there would not be any block to the influx of either lactose as a substrate for β-galactosidase or methyl-methane sulfonate as an inducer of the SOS response.

There is another method for the *B. subtilis* permease to affect solute influx. The solute:cation symport system may be blocked because the chemiosmotic gradient, necessary for the transport of a variety of solutes, has been destroyed (9). The overexpression of the *B. subtilis* permease may tax the ability of the cell to also import sugars such as lactose, which functions as a solute:cation symport system, or those necessary for the production of the colanic acid polysaccharide. This may also affect the importation of methyl-methane sulfonate and subsequent induction of the SOS response. The apparent phenotype would be of a lon^+ cell while the actual reason for it would not be the production of a Lon-like protease.

The function of ORF5 is unknown in *B. subtilis*. ORF5 does not have any significant homology to sequences present in the DNA or protein databases (29). According to the published sequence of ORF5, it is located chromosomally upstream of the pyruvate dehydrogenase (*pdh*) operon. The mRNA for the ORF5 gene is transcribed from its own promoter, independent of the three promoters found in the *pdh* operon (29). The lack of information concerning ORF5 function prompted a re-examination of its role in suppressing the mucoid phenotype, in turning off *cps::lacZ* expression, and in restoring resistance to DNA damaging agents in *E. coli* Δ*lon* mutant cells. The expression of the mucoid phenotype is reported to be regulated by three mechanisms: degradation of a transcriptional activator (RcsA) by a protease (Lon), activation of a second transcriptional activator (RcsB) by a membrane bound sensor (RcsC), and interaction of the two transcriptional activators (RcsA, RcsB) at the *cps* promoter (23). Thus, ORF5 activity may be suppressing the mucoid phenotype by repressing expression of RcsA or RcsB, interfering with the interaction of RcsB with RcsC, interfering with the interaction of RcsB with RcsA, or interfering with the interaction of RcsA with the *cps* promoter. The ORF5 activity encoded by pJET39.20 did not repress expression of either RcsA or RcsB, nor did it interfere with the interaction of RcsB with RcsC. ORF5 activity did suppress the mucoid phenotype of an *E. coli* strain that

synthesized a RcsA protein resistant to Lon degradation (SG4147). This discovery casts doubt to the possibility that ORF5 acts as a protease to suppress the mucoid phenotype, and it suggests that ORF5 effects are the result of interference with the interaction of RcsA with RcsB or the interaction of RcsA with the *cps* promoter.

How does this information extend to explain the ORF5 role in restoring an appropriate SOS response to DNA damaging agents such as methyl-methane sulfonate? The expression of the Lon substrate, SulA, occurs in response to DNA damage. SulA acts a cell division inhibitor which blocks septation by interacting with FtsZ, a component of the cell division apparatus (32, 33). Lon protease cleaves SulA during the SOS response allowing cell division to resume after DNA repair has taken place (25). In the absence of Lon protease, SulA accumulates and leads to a lethal filamentation phenotype in *E. coli*. The activity encoded by ORF5 does not repress expression of SulA, however, this activity may interfere with the interaction of SulA with FtsZ, leading to a lon^+ phenotype to *E. coli* Δ*lon* cells during the SOS response. As noted in Figure 2, *ftsZ* expression is activated by RcsB (25). Since cell division is not blocked, binding of SulA with FtsZ must be hindered, perhaps by a method suggestive of the case of RcsA with RcsB. ORF5 mode of action may be to bind to proteins, thus preventing necessary interactions with other proteins that lead to either capsule polysaccharide synthesis or inhibition of cell division in *E. coli*.

Summary.

Two fragments of *Bacillus subtilis* DNA encode activities that suppress the phenotypes of mucoidy and sensitivity to DNA damaging agents in *Escherichia coli* Δ*lon* cells. One DNA fragment expresses a protein comparable in size to the gluconate permease. Presumably this enzyme suppresses the *lon* phenotype by blocking transport of lactose (for *cps::lacZ* evaluation), methyl-methane sulfonate (inducer of SOS response), and components necessary for capsule synthesis (e.g. sugars). The other DNA fragment expresses a protein comparable in size to an open reading frame of

unknown function named ORF5. Presumably this protein suppresses the *lon* phenotype by interfering with a regulatory protein's interaction with either another protein or a DNA sequence

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BIOGRAPHICAL INFORMATION

Donald Darwin Chen was born May 19, 1949 in Washington, DC to Huan-Yung and Suzy Wang Chen. He was raised in New York City and went to school in New York and Connecticut. He started his undergraduate studies in mathematics at Northeastern University (Boston), but left to pursue other interests reflective of the 1960s. He received an AS in Livestock and Dairy Technology from Clatsop Community College (Astoria, OR) in 1979, and a BS in Animal Science from Oregon State University (Corvallis) in 1981. He has worked in laboratories studying baculoviruses and fish immunology before starting work with Dr. Janine Trempy in the field of bacterial genetics.