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Inducible Error-prone Repair in *B. subtilis*

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DISCLAIMER

ABSTRACT

The research performed under this contract has been concentrated on the relationship between inducible DNA repair systems, mutagenesis and the competent state in the gram positive bacterium Bacillus subtilis. The following results have been obtained from this research:

I. Competent Bacillus subtilis cells have been developed into a sensitive tester system for carcinogens;

II. Competent B. subtilis cells have an efficient excision-repair system, however, this system will not function on bacteriophage DNA taken into the cell via the process of transfection;

III. DNA polymerase III is essential in the mechanism of the process of W-reactivation;

IV. B. subtilis strains cured of their defective prophages have been isolated and are now being developed for gene cloning systems;

V. Protoplasts of B. subtilis have been shown capable of acquiring DNA repair enzymes (i.e., enzyme therapy); and

VI. A plasmid was characterized which enhanced inducible error-prone repair in a gram positive organism.

SCIENTIFIC BACKGROUND

Errorprone vs. Errorfree DNA Repair Systems:

The discovery that certain ultraviolet (UV) sensitive mutants of Escherichia coli are not mutated following UV radiation led to the hypothesis of error-prone and error-free types of DNA repair systems (1-6). Essentially, mutagenesis at the level of the DNA occurs via two distinct mechanisms: (i) mispairing during DNA replication and (ii) misrepair or mistakes during the repair of damaged DNA. It is this later forof mutagenesis which has been called an error-prone mechanism of DNA repair. Classically, DNA repair mechanisms can be divided into three main components (photoreactivation, exicision-repair and post-replication repair).

Photoreactivation:

Photoreactivation, is a repair mechanism which acts specifically on the pyrimidine dimers produced following UV radiation (7,8,9). The photoreactivating enzyme binds to the dimer and then 'splits' the dimer following the exposure of the enzyme-dimer complex to photoreactivating light (8,9). This mechanism of DNA repair is error-free since it does not result in the production of mutations (3,10). Photoreactivation does not exist in the bacterium Bacillus subtilis. Therefore, this type of DNA repair mechanism should be of limited importance for this research. However, our laboratory is attempting to clone into B. subtilis the gene which codes for this enzyme.

Excision-Repair:

Damaged DNA can also be repaired in the dark via the excision mechanism (3,11, 12). This type of repair was generally believed to be error-free (2,3,10,13,14). The original concept of excision repair involved a system which consisted of four steps: an endonucleolytic nick was made in the vicinity of the damage; the damaged section was removed by an exonuclease; the resulting gap filled in by a DNA polymerase (the DNA strand opposite the damage was used as a template); and the new stretch of DNA joined to pre-existing DNA by ligase (11). In E. coli the most efficient and accurate excision repair system requires, among other gene products, a functional DNA polymerase I (15,16,17). However, alternate pathways of excision repair have been demonstrated in cells lacking DNA polymerase I (3, 18,19,20). These alternate pathways of excision repair have been associated with error-prone components (21,22). Also, in Micrococcus luteus and bacteriophage T4-infected E. coli, the UV excision repair system has been shown to involve glycosylase (23,24). These glycosylases cause the rupture of the glycosylic bond between one of the pyrimidines of the dimer and the deoxyribose sugar leaving an apyrimidinic site (23,24,25). The apyrimidinic site is then cleaved by an AP (apurinic or apyrimidinic) endonuclease (23,24,26). This type of excision repair is analogous to the 'base excision repair' systems which remove altered bases in the DNA of different organisms (27,28).

The existence of error-prone excision repair as well as the involvement of glycosylases in some (if not all) excision repair systems has caused a surge or renewed interest into the mechanisms which provide this type of DNA repair. The recent observations cited above were certainly not predicted by the early models of excision repair. The continued investigation of the mechanisms of DNA repair in a variety of organisms facilitated the discovery of those unexpected results.

Interestingly, it has been recently shown that components of the excision-repair system, in E. coli are inducible (the proteins are not constitutitively produced in the cell; 18,29,30). This finding was again not predicted by the classical models of excision repair. Inducible types of DNA repair systems had previously been thought of as being part of post-replication repair (3-6).

Post-Replication Repair and 'SOS' Phenomena:

Post-replication repair is believed to involve the filling in of gaps left in daughter strand DNA following the replication of DNA which has been damaged (3,31,32). The closing of these single strand gaps is accomplished by at least two types of mechanisms in E. coli (33). First, a constitutive type of post-replication repair has been shown to involve recombination (34,35,36). In addition, one or more types of post-replication repair are inducible and their mechanisms of action are unknown (37,38). Post-replication repair in E. coli (and some other organisms) has an error-prone element (1,3,37,39,40), which is an inducible independent minor pathway. Specifically, mutants of E. coli which are defective in recA$^+$ or lexA$^+$ gene products are unable to induce this error-prone repair system (3,41,42). In addition to error-prone inducible post-replication repair, recA and/or lexA (exrA) mutations prevent error-prone excision repair (18), induction of certain prophages (43,44), the induction of the recA$^+$ gene product [formerly called protein X; (3,45,46,47)] the inhibition of exonuclease V (48), W-or UV reactivation, and W-mutagenesis (3,41,49,50), as well as other physiological changes following the inhibition of DNA replication and/or damage of the DNA (51). The pleiotropic effects of these mutations led Radman (52) to propose the 'SOS' hypothesis. This theory contends that damage to DNA and/or the inhibition of DNA replication results in the release of a signal which simultaneously activates various functions which aid the cells and/or prophages in survival (3,52). Therefore, error-prone repair is the result of an efficient but inaccurate repair mechanism which is induced in an effort to prevent cell death.

Regulation of the 'SOS' system:

If the 'SOS' hypothesis is correct, then this system must play an important role in mutagenesis and/or cell survival. Therefore, this hypothesis should be tested and a complete understanding of how the 'SOS' system functions must be obtained. The recA$^+$ gene product has been shown to be involved in recombination and repair (3,46,47,53, 54,55). This protein is produced in small quantities constitutively by E. coli. Following activation it is believed that the recA$^+$ product is altered such that it now has proteolytic activity (46,56,57). This protease is capable of acting on the lambda repressor (thus causing the induction of prophage lambda) and on the lexA$^+$ gene product (46,48,49). The lexA$^+$ gene product is a repressor for the recA$^+$ and lexA$^+$ genes (58,60). Thus the activation of recA$^+$ protein into its proteolytic form results in the cleavage of the lexA$^+$ protein and subsequently into the production of increased levels of recA$^+$ and lexA$^+$ proteins. The increased production of lexA$^+$ protein eventually results in the restoration of the repression of the recA$^+$ gene after the DNA damage has been repaired (58). Potentially the lexA$^+$ product could be a repressor for other 'SOS' functions and/or the recA$^+$ product could have proteolytic activity on additional repressors. For instance, W-reactivation (the increased survival of DNA damaged bacteriophage when they are grown on DNA damaged bacteria as compared

to when they are grown on bacteria not having damaged DNA) could be the result of the production of a new or altered type of DNA polymerase. It has been proposed that the induction of the 'SOS' system results in either the production of a new DNA polymerase or the alteration of the 'editing' activity of DNA polymerases (52, 61,62). This proposal has been made in an attempt to explain why the W-reactivation of bacteriophage also results in an increased mutation frequency among the W-reactivated viruses (W-mutagenesis; 3,50,52). Similarily, these less accurate polymerases could also explain chromosomal error-prone DNA repair (3,52). In fact, W-reactivation, W-mutagenesis, and error-prone repair have all been proposed to be manifestations of the same molecular mechanism. Support for this hypothesis can be inferred from the isolation of a mutation (umuC) which abolishes only these three components of the 'SOS' system (63). Bridges et al., (64,65) have suggested that DNA polymerase III is intricately involved in error-prone repair, while Radman and his colleagues have presented data which indicates that 'SOS' induced cells are enhanced in their ability to replicate UV irradiated single strand bacteriophages (66). This later data would seem to imply that a DNA polymerase in 'SOS' induced cells is capable of bypassing pyrimidine dimers. This type of activity could explain W-reactivation, W-mutagenesis and error-prone repair. Unfortunately, an altered DNA polymerase has not yet been obtained from 'SOS' induced cells. Therefore, the evidence which indicates DNA polymerase involvement in error-prone repair is primarily genetic in origin.

In addition to the lack of an isolated biochemically altered DNA polymerase from 'SOS' induced cells, it is still not understood how the activity of the recA$^+$ gene product is changed such that it begins to function as a proteolytic enzyme (67). However, nucleotides have been implicated as inducers for the activation of the 'SOS' system (62,67,68). In any event, further study of the tif-1 mutation (an allele of the recA gene) may yield some insight on how the recA$^+$ protein is regulated. The tif-1 mutation allows for the activation of the recA protease at elevated temperatures without damage to the organism's DNA (69).

The vast majority of work on the 'SOS' system has been done in the bacterium E. coli. The existence of such an inducible system would seem to represent an important evolutionary development. This type of inducible system would provide not only an additional mechanism for survival, but possibly even more significant, a method for increasing the genetic variability of a species. Therefore, it is not surprising to find a wide range of organisms in which some part of the 'SOS' system has been identified (reviews; 3,4,70).

The Developing 'SOS' Hypothesis:

Additional investigation of 'SOS' related phenomena as well as investigations into other types of inducible repair systems has resulted in substantial changes in the original 'SOS' concept. Specifically, error-free as well as error-prone inducible repair components of the 'SOS' system have been identified (71,72) and an inducible error-free repair system has been identified which is not dependent on recA$^+$ gene activity (73,74). Also, plasmids have been identified which increase the UV resistance and UV induced mutation frequency of bacteria (75,76). The most extensively studied of these plasmids, pKM101, has been shown able to circumvent

mutations in the chromosomal umuC gene (77). Also, the enhanced resistance and the increased mutation frequency which these plasmids confer upon the bacteria in which they reside depend on functional recA$^+$ and lexA$^+$ gene products (76,78). The results described above would appear to suggest that the plasmids are carrying umuC$^+$ genes. However, analysis of the types of mutations obtained in plasmid carrying strains and in plasmid free strains have clearly demonstrated that the chromosomal error-prone system and the plasmid error-prone system are different (79,80,81).

'SOS' Functions and Cancer; A Possible Relationship:

Significantly, interest has also begun to focus on the possible relationship between mammalian 'SOS' systems and the development of cancer in animals (3,82). In the tester system developed by Bruce Ames and his colleagues (83,84), most carcinogenic agents have been shown to require a functional error-prone repair system in order to generate mutations in the bacteria (84). In addition, known carcinogenic agents induce prophage lamda and therefore activate the bacterial 'SOS' system (85,86). Additional tester systems have been developed which utilize the induction of 'SOS' functions as an indication of potential carcinogenic activity (82,87,88). Based on the positive results from these tests, it has been speculated that 'SOS' systems (or at least similar systems) exist in eukaryotes, and that the abnormal activation of these systems can lead to cancer (3,52,82). If this theory is correct, DNA damage in mammalian cells should result in the induction of physiological phenomena similar to that which occurs in E. coli (i.e., error-prone inducible repair, induction of viruses, W-reactivation, and W-mutagenesis). Both error-prone and inducible post-replication repair have been inferred to occur in mammalian cells in culture (88,89,90). Furthermore, there appears to be reactivation of irradiated human viruses following the irradiation of host cells (92,93); a process similar to W-reactivation. In addition, damage of mouse cell DNA, by certain agents, can lead to the induction of viruses which are resident in the chromosomes of these cells (98,93); a process analogous to the induction of prophage. In summary, the evidence suggests that an 'SOS' like system exists in eukaryotes and this system may be involved in development of neoplastic events.

'SOS' Phenomena in B. Subtilis:

Of special interest to this research is the association of the 'SOS' system and the state of competency in the bacterium Bacillus subtilis. Competency is the ability of an organism to bind and utilize (in its genome) exogenous DNA (96). Our laboratory has postulated that when B. subtilis differentiates into its competent state, there is a precocious activation of the bacterial 'SOS' system (97). Support of this theory has been obtained from certain experimental results (3,98,99,100). Specifically, evidence has been presented which shows the

existence in B. subtilis of an inducible system analogous to the 'SOS' system of E. coli (101). In B. subtilis, 'SOS' functions so far identified consist of prophage induction, W-reactivation, the induction of a DNA modification system (recently shown to be a function of prophage SPβ;102,103), the appearance of a protein (possibly analogous to the recA$^+$ gene product), and an inducible form of DNA repair. Additionally, error-prone repair has been suggested for B. subtilis (100), and mutants of B. subtilis have been characterized in which the activation of the 'SOS' functions do not occur (97).

Competent B. subtilis cells are more sensitive to 'SOS' inducing agents than are non-competent cells (83,99). This enhanced sensitivity is abolished if the resident prophages are removed from the chromosome of B. subtilis (83,99). Essentially, the competent cells are more sensitive to 'SOS' inducing agents because of the precocious activation of 'SOS' functions. "Precocious activation" is meant to signify that the threshold for the induction of 'SOS' functions is lowered in competent cells (87,100). Additionally, competent bacteria show the spontaneous activation of a prophage controlled DNA modification system (99,102), and they have an increased mutation frequency (98, 105). Thus, the data indicate that the 'SOS' system is activated ("Precocious activation") when B. subtilis becomes competent.

If the model suggested in the previous paragraph is correct, the 20% of a culture of B. subtilis which can become competent (106), have increased their genetic variability by inducing the 'SOS' system (error-prone repair). Such a controlled increase in genetic variability would be evolutionarily beneficial to the bacterial species. However, if the majority of the population spontaneously induced an error-prone repair system, then the bacterial culture would suffer from the increased mutation frequency and the resulting genetic load. Therefore, one would speculate that in bacteria where the majority of the population become competent, spontaneous activation of the 'SOS' system would not occur or the 'SOS' system would be altered such that there might not be an error-prone repair element. Interestingly, in Streptococcus pneumoniae (106), Haemophilus influenzae (108) and Neisseria gonorrhea (109) where a majority of the cells become competent (110), there appears to be no error-prone mechanism of DNA repair. However, in Haemophilus, W-reactivation has been identified although W-mutagenesis is absent (117). Although the evidence is circumstantial, it is possible that the 'SOS' systems of competent bacteria have been modified to play some important role in the recombinational events which are part of the DNA-mediated transformation process.

Results Obtained:

Our laboratory has been interested in the molecular mechanisms responsible for inducible DNA repair systems and the relationship between the 'SOS' system and competence development in Bacillus subtilis. The following is a brief review of the results which have been obtained during the past three years:

I. Competent *Bacillus subtilis* cells are a sensitive tester system for carcinogens. The development of competent transformed Bacillus subtilis into a tester system for carcinogens has been achieved (87). Precocious activation or non-induced activation of 'SOS' functions occur in competent B. subtilis (99).

Thus, lower doses or concentrations of 'SOS' inducing agents are needed to cause cell death due to indigenous prophage activation and lysis of bacteria in competent cells as compared to non-competent cells. The two known defective prophages in B. subtilis enhance the sensitivity of competent cells to the carcinogens ultraviolet light, mitomycin C, and methyl methanesulfonate. However, these same cells have no enhanced sensitivity for the non-carcinogenic (or weakly carcinogenic) ethyl methanesulfonate or for nalidixic acid (etc.). Therefore, competent B. subtilis appear to be a sensitive tester for carcinogens (Comptest). The Comptest has been used, along with the Ames Salmonella Assay, to determine the mutagenic and DNA damaging capacity of potential antitumor drugs (111), environmental pollutants and the particulate isolated from the exhaust of Diesel engines (112, 113, 114).

II. Excison Repair Capacity of Competent *Bacillus subtilis*. Competent B. subtilis cells were investigated for their ability to support the repair of UV-irradiated bacteriophage and bacteriophage DNA (100). UV-irradiated bacteriophage DNA cannot be repaired as efficiently as can UV-irradiated bacteriophage. This result suggested a deficiency in the ability of competent cells to repair UV damage since only the bacteriophage DNA was being assayed on competent cells. However, competent cells were as repair proficient as noncompetent cells in their ability to repair irradiated bacteriophage in marker rescue experiments. The increased sensitivity of irradiated DNA was shown to be due to the inability of the excision repair system to function on transfecting DNA in competent bacteria. This conclusion was based on data which demonstrated that UV irradiated transfecting bacteriophage DNA was not repaired by the excision repair system of competent B. subtilis (100) but was repaired by the excision repair system of protoplasts of B. subtilis.

III. Role of DNA Polymerase III in W-Reactivation in *Bacillus subtilis*. 6-(p-hydroxyphenylazo)-uracil, a purine analog that is known to specifically inhibit deoxyribonucleic acid polymerase III in gram positive organisms, inhibited W-reactivation in Bacillus subtilis (115). On the other hand, W-reactivation proceeded normally in the presence of 6-(p-hydroxyphenylazo)-uracil when a strain possessing a resistant DNA polymerase III was used as the host. Significantly, the bacteriophage used in this study was not inhibited in its own replication by the antibiotic. Also, the antibiotic did not interfere with the induction of the bacterial 'SOS' system. Thus, the data clearly demonstrated that DNA polymerase III is an essential enzyme in the mechanism of action of W-reactivation in the bacterium B. subtilis.

IV. Establishment of *Bacillus subtilis* Strains Cured of their Defective Prophages. During the last two and a half years certain difficulties have been encountered in attempts to characterize the mechanism of UV mutagenesis in B. subtilis. A by-product of attempts to establish the best B. subtilis strain in which to do mutagenesis has been the development of an isogenic set of strains which should aid in the utilization of B. subtilis as a host for recombinant DNA cloning (102). These isogenic strains are non-inducible for prophage PBSX and are cured of prophage SPβ. These strains were originally designed to prevent prophage induced lysis of the bacteria following the UV irradiation of B. subtilis. It was postulated that part of the problem which was being encountered in the isolation of UV-induced mutants could be explained by the presence of these two prophages in the chromosome of B. subtilis (i.e., the mutants that were being induced were being lysed).

Analysis of the genetic properties of these prophage cured strains revealed that prophage SPβ controlled the inducible DNA modification enzyme which is a component of the 'SOS' system of B. subtilis (102). However, neither the PBSX nor the SPβ prophages alter the ability of the bacterium to undergo genetic recombination, to repair damaged DNA, nor to sporulate (102). Prophageless B. subtilis strains will probably be useful hosts for the ϕ3T cloning vector (which is being developed in several laboratories), because of the absence of vector-SPβ prophage interactions in these strains (Zahler, personal communication; Weiner and Yasbin, manuscript in preparation).

V. Plasmid Enhancement of UV Mutagenesis in Gram Positive Bacteria. In addition to directly examining the question of whether or not error-prone repair exists in B. subtilis, experiments were begun to determine if it would be feasible to introduce an error-prone repair system into this bacterium from another organism. Specifically, a 38.5 Mdal plasmid of Streptococcus faecalis subsp. zymogenes was shown to enhance survival following UV irradiation. In addition, the presence of this plasmid increases the mutation frequencies following UV irradiation and enhances W-reactivation of UV irradiated bacteriophage (116). The data indicate that S. faecalis has an inducible error-prone repair system and that the plasmid enhances these repair functions or adds additional error-prone repair functions. Our laboratory will attempt to introduce this plasmid or a similar plasmid into B. subtilis in order to establish or enhance error-prone repair in this organism.

VI. The Acquisition of DNA Repair Enzymes by Protoplasts of *B. subtilis*. A novel form of 'enzyme therapy' was achieved by utilizing protoplasts of B. subtilis. Photoreactivating enzyme of E. coli was successfully inserted into the protoplasts of B. subtilis which had been treated with polyethylene-glycol. This enzyme was used to photoreactivate UV damaged bacteriophage DNA. The methodology used in these experiments can be adapted in order to identify and characterize additional DNA repair enzymes.

Preprints + Reprint Request

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