Mutations showing specificity for normal growth or Mn(II)-dependent post-exponential-phase cell division in *Deinococcus radiodurans*

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We have previously reported that in the presence of small amounts of Mn(II) ions, stationary-phase cultures of the radioresistant bacterium *Deinococcus radiodurans* IR are capable of undergoing about three new rounds of cell division which are non-exponential and reductive. This novel Mn(II)-induced cell division (Mn-CD) phenomenon was studied further. Two mutants were isolated from *D. radiodurans* IR and were found to harbour mutations affecting the continuation of cell division and DNA replication, one of which affected vegetative growth but not Mn-CD, while the other affected Mn-CD but not vegetative growth. Moreover, a DNA synthesis transition was observed when growth was switched from the normal to the Mn-CD process. These results suggest the presence in *D. radiodurans* of an alternative type of cell division induction, which is characterized by being Mn(II)-dependent and stationary-phase specific.

Keywords: *Deinococcus radiodurans*, cell division, manganese(II), temperature-sensitive mutant

INTRODUCTION

The members of the genus *Deinococcus*, a phylogenetic outliner among bacteria (Woese, 1987), are known for their extreme resistance to the lethal effects of UV (254 nm) and ionizing radiations (Anderson *et al.*, 1956; Moseley, 1983; Murray, 1992).

Regarding this radioresistance, the divalent cation Mn(II) is of particular interest. It has been reported that Mn(II) is bound to chromosomal DNA in large amounts in *Deinococcus* (formerly *Micrococcus*) *radiodurans* (Leibowitz *et al.*, 1976), is required for the activity of a DNA repair enzyme (Evans & Moseley, 1985), and is involved in the high radioresistance in this bacterium (Wierowski & Bruce, 1980). Recently, we found that addition of Mn(II) at 2.5 µM or higher to stationary-phase cultures of *D. radiodurans* IR was capable of triggering new rounds of cell division (Chou & Tan, 1990). This cell division induction was termed 'Mn(II)-induced cell division' (Mn-CD). Cells in the Mn-CD stage lose resistance to both UV and gamma radiations as a function of the number of Mn-CDs (Chou & Tan, 1990). In addition to the increased sensitivity to radiation-induced cell inactivation, physio-

logical changes accompanied by the Mn-CD effect include a shortened stationary phase before rapid death of the culture as well as an increase in both superoxide dismutase and catalase activities. Mn-CD apparently results from a change in growth control by either disrupting or bypassing a cell division arrest (Chou & Tan, 1990). The Mn-CD effect appears to be Mn(II) and *Deinococcus*-specific. Little is, however, known about the mechanism(s) involved.

The nature of Mn-CD is further clarified and discussed in this report. Cell division cycle mutants, including a temperature-sensitive mutant and an Mn-CD-negative mutant, have been isolated in this study. The physiological and genetic analyses of these different cell division cycle mutants suggest the presence in *D. radiodurans* of an alternative pathway in the cell division cycle which is activated by Mn(II) and operates only in the stationary phase; that is, the pathways of normal cell division (N-CD) and Mn-CD appear to be separable in certain step(s).

METHODS

Strain, media, and growth conditions. *D. radiodurans* IR, formerly *Micrococcus* isolate C-7, has been characterized previously (Tan, 1982; Tan & Maxcy, 1982, 1986; Chou & Tan, 1990, 1991). Growth conditions were described previously (Chou & Tan, 1990). Unless otherwise specified, cell cultivation

Abbreviations: Mn-CD, Mn(II)-induced cell division; N-CD, normal cell division.

was at 32 °C. Liquid cultivation was performed routinely with shaking at 160 r.p.m. in plate count broth (PCB; Difco) containing 5 g yeast extract, 10 g tryptone, and 2 g dextrose l⁻¹. Plate count agar (PCA; Difco) was used in cell enumeration by the standard plate count method as previously described (Tan & Maxcy, 1982). Phosphate buffer (67 mM, pH 7.0) was used for washing, suspending and diluting bacteria. MnSO₄·H₂O (E. Merck) of analytical grade was used to prepare the Mn(II) solution.

Determination of cell size. Cell samples appropriately diluted with an electrolyte solution (ISOTON II, Coulter Electronics) were analysed for cell size by using a particle analyser (Coulter Multisizer II, Coulter Electronics) (Kubitschek, 1990).

Test for Mn-CD effect. Overnight culture (0.1 ml) was transferred to two test-tubes (each containing 10 ml PCB) and incubated until reaching the early stationary phase. Mn(II) was added to one of the tubes at 10 µM, and both of the tubes were further shaken for 12 h (for a full expression of Mn-CD). A higher turbidity in the Mn(II) tube due to reductive cell divisions was regarded as a result of the Mn-CD effect.

Isolation of mutants. For all mutagenesis treatments, the late-exponential-phase cells were washed and resuspended in phosphate buffer before adding *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [NTG, dissolved in dimethyl sulfoxide/phosphate buffer (3:7, v/v)] at dosages causing an approximate 50% reduction in cell viability.

(i) **Temperature-sensitive mutant.** NTG-treated *D. radiodurans* IR cells were washed twice with phosphate buffer, resuspended in PCB, and incubated overnight. The culture was then properly diluted and plated on PCA to yield 100–200 colonies per plate after incubation for 2–3 d. Discrete colonies were transferred with a toothpick onto PCA plates such that two replicate copies of each plate were made, one of which was incubated at 32 °C and the other at 39 °C. After overnight incubation, colonies which grew at 32 °C, but not at 39 °C, were isolated and tested to verify the temperature sensitivity.

(ii) **Mn-CD-negative mutant.** To increase the chance of isolating Mn-CD-negative mutants of interest, two enrichment steps were designed (because no information concerning the Mn-CD phenomenon and its genetic background is available). The first of these was enrichment of mutants by heat treatment. The Mn-CD cells of *D. radiodurans* IR were previously found to be more sensitive to a lethal temperature than untreated control cells. When NTG-treated cells were grown in PCB with 10 µM Mn(II) until reaching the end of Mn-CD, and heated at 60 °C for 20 min, Mn-CD-negative mutants would be enriched. The second enrichment step involved distinction of colony size on Mn(II)-supplemented agar plates. As it lacks Mn(II)-mediated 'extra' cell divisions, the Mn-CD-negative mutant would form colonies smaller than that of the wild-type (Mn-CD positive) strain on Mn(II)-containing agar plates. Thus, heat-treated cells were spread on PCA containing 10 µM Mn(II). Colonies with a relatively small size on PCA would be picked up and further tested for the Mn-CD effect (see earlier).

Measurement of DNA synthesis. A modified method of that described by Boylan & Mendelson (1969) was used to measure DNA synthesis. Briefly, [*methyl*-³H]thymidine [6.7 Ci mmol⁻¹ (247.9 GBq mmol⁻¹)] was added to give a final activity of 20 µCi ml⁻¹ (740 kBq ml⁻¹) to the cultures grown in PCB supplemented with 100 µg thymidine ml⁻¹. Samples (0.1 ml) were removed at various times and added to 10 ml ice-cold 10% (v/v) TCA. The acid-insoluble fraction was collected by centrifugation (6000 r.p.m.; 5 min) and washed separately three times with 10 ml 5% (v/v) TCA. The radioactivity in the acid-

insoluble fraction suspended in 3 ml of a scintillation counting solution was determined with a liquid scintillation counter.

RESULTS AND DISCUSSION

Isolation of a temperature-sensitive mutant that fails to grow, but retains Mn-CD demonstrability, at the restrictive temperature

After an NTG (0.5 g l⁻¹) treatment, a mutant that grew at 32 °C but not at 39 °C was isolated from about 5000

Fig. 1. Growth and [³H]thymidine incorporation (inset) of *D. radiodurans* IR (a) and its temperature-sensitive mutant TS1 (b). Cultures were grown in PCB and the temperature was shifted to 39 °C (□) after 3 h at 32 °C (○) as indicated by arrows. Data for growth measurement are means of at least two replications. Individual points of radioactivities represent the mean of three independent measurements differing by less than 10%.

Fig. 2. Mn-CD of stationary-phase TS1 cells at the restrictive temperature (39 °C). Cultures were pregrown at 32 °C to the stationary phase in PCB, and the temperature was then shifted to 39 °C as indicated by the large arrow. ○, 32 °C; □, 39 °C; ■, 39 °C with Mn(II). The times of Mn(II) addition are indicated by the small arrows. Data are means of at least three replications.

survivors. Growth and DNA synthesis of this mutant (TS1) in PCB were immediately halted following the temperature shift from 32 °C to 39 °C, whereas those of IR remained unaffected (Fig. 1). Evidently, TS1 was a temperature-sensitive mutant whose N-CD pathway was blocked at the restrictive temperature (39 °C). Interestingly, the stationary-phase cultures of TS1 (grown at 32 °C) could undertake further cell division at not only 32 °C (data not shown) but also 39 °C (Fig. 2) upon the addition of 10 µM Mn(II). This suggests that the Mn-CD and the N-CD pathways appear to be separable from each other. These results also suggest that in TS1 the temperature-sensitive mutation(s) might be in a gene coding a specific component needed for DNA synthesis and expressed in exponential-phase cells. This specific component is essential for the N-CD process, whereas it is not involved in, nor essential for, the Mn-CD process. We propose the presence of an Mn(II)-dependent component that is required for DNA synthesis of Mn-CD process in stationary-phase cells of *D. radiodurans* IR.

Additionally, it was noted that the wild-type strain and TS1 both showed earlier transition from stationary phase into death phase at 39 °C, with or without added Mn(II) (TS1 data shown in Fig. 2).

The Mn-CD mechanism is activated only when a culture has entered the stationary phase

To determine whether activation of the Mn-CD mechanism could occur in an exponential-phase culture of TS1, an overnight culture of TS1 was inoculated into PCB at a low initial cell density (approximately 10^3 c.f.u. ml⁻¹) and then cultivated at 32 °C. Cultures at the mid-exponential, late-exponential and early-stationary phases (approximately 5×10^6 , 5×10^7 , and 2×10^8 c.f.u. ml⁻¹, respectively)

Fig. 3. Effects of temperature shift from 32 °C (○) to 39 °C (□, ■) on growth (a) and [³H]thymidine incorporation (b, c) of the exponential-phase (A, B) and stationary-phase (C) cultures of a temperature-sensitive mutant TS1 with (■) or without (□) Mn(II) addition. Parts (b) and (c) show the incorporation data from the cultures represented by A and C in part (a), respectively. Arrows indicate the times of temperature shift plus Mn(II) addition. Data for growth measurement are means of at least two replications. Individual points of radioactivities represent the mean of three independent measurements differing by less than 10%.

ively) were each treated with Mn(II) at 10 µM and shifted to 39 °C. Samples were then taken at intervals for determining the further cell division and DNA synthesis. The results indicated that the stationary-phase cultures could demonstrate Mn-CD (Fig. 3a) and DNA synthesis (Fig. 3c) at 39 °C, whereas both the mid- and late-exponential phase cultures [in a sense these cultures entered a stationary (more accurately, static) phase after shifting to 39 °C] could not (Fig. 3a, b). These results suggest that only the cells physiologically entering the stationary phase are capable of demonstrating the Mn-CD

Fig. 4. Growth of *D. radiodurans* IR (a) and its Mn-CD-negative mutant D1 (b) in PCB at 32 °C (○) and their responses to Mn(II) addition (●) in the stationary phase. Arrows indicate the times of Mn(II) addition. Data are means of at least two replications.

phenomenon, while the exponential-phase cells physically forced to enter a nongrowing phase are not. It appears that the Mn-CD pathway is normally blocked in the exponential phase and that some gene(s) is activated to initiate Mn-CD in the stationary phase, whereas the N-CD pathway would prevail in the exponential phase.

Certain cellular proteins were found to increase with time of Mn-CD (Chou & Tan, 1990). Mn(II) may be involved in an initiation process of cell division; it either induces *de novo* protein synthesis or causes activity changes in pre-existing stationary-phase specific protein(s). Stationary-phase specific proteins have been shown in *Escherichia coli* (Groat *et al.*, 1986; Jenkins *et al.*, 1990; Siegle & Kolter, 1992), *Salmonella typhimurium* (Spector *et al.*, 1986) and *Vibrio* sp. (Nyström *et al.*, 1990). The Mn-CD effect presents a novel example to stress the generality of stationary-phase phenomology; other examples include sporulation in *Bacillus subtilis* (Nicholson & Setlow, 1990; Oke & Losick, 1993), production of secondary metabolites in *Streptomyces* spp. (Basarán *et al.*, 1991;

Gramajo *et al.*, 1993), and storage metabolism in *Saccharomyces cerevisiae* (Lillie & Pringle, 1980).

The Mn-CD mechanism is not essential for cell viability and multiplication

As indicated above, DNA replication of the N-CD but not Mn-CD process in TS1 was blocked at 39 °C, suggesting that the Mn-CD process possibly involves an alternative DNA replication pathway. Therefore, blocking the Mn-CD-dependent pathway would not be lethal to the cell, and it should be possible to isolate mutants which are defective in Mn-CD.

About 20000 NTG-mutagenized IR cells were enriched for Mn-CD-negative mutants. Finally, about 2000 colonies were subjected to the Mn-CD test and an Mn-CD-negative mutant was isolated. This mutant (D1) displayed Mn-CD negativity at a wide range of Mn(II) concentration (1–500 µM). It had wild-type colony size, doubling time, and tetracoccal form, but exhibited a significantly lower maximum growth (in c.f.u.ml⁻¹), only 1/3–1/2 that of IR. Growth curves of IR and D1 in PCB at 32 °C, with or without Mn(II) addition, are illustrated in Fig. 4. Mn-CD was not observed in D1 (Fig. 4b), indicating a block in the Mn-CD pathway in this mutant. The D1 mutant phenotype was the same at 25 °C, 32 °C, and 39 °C, and its general growth characteristics were similar to those of IR. Therefore, D1 is not a temperature-sensitive mutant; it may be defective in certain gene(s) involved in the Mn-CD pathway, while the gene(s) is not essential for the N-CD pathway. The successful isolation of such an Mn-CD-negative mutant which differs from the wild-type (IR) specifically in post-exponential physiology supports the inference that the Mn-CD pathway is not essential for cell viability and multiplication in *D. radiodurans*.

The Mn-CD-negative mutation(s) blocks DNA replication

The Mn-CD-negative mutant D1 was compared with the wild-type strain IR in capability of Mn uptake. Early-stationary-phase cells grown in PCB plus 50 µM Mn(II) were harvested and subjected to inductively coupled plasma atomic emission spectrometry analysis for intracellular Mn concentration by a procedure described by Hawke & Lloyd (1988). The results showed D1 cells contained $4.5 \pm 0.9 \times 10^{-18}$ mol Mn c.f.u.⁻¹, which was comparable to that of IR ($4.7 \pm 1.1 \times 10^{-18}$ mol Mn c.f.u.⁻¹). Therefore, D1 was not defective in Mn uptake. In another experiment, an early-stationary-phase D1 culture was dispensed into two tubes: 10 µM Mn(II) was added to one, then the tubes were incubated as usual. Cell sizes were monitored by a Coulter counter for up to 4 h. The sizes of Mn(II)-treated D1 cells were found to be comparable to those of Mn(II)-untreated cells (e.g. at 1.5 h, the sizes were $9.8 \pm 0.7 \mu\text{m}^3$ and $9.7 \pm 0.6 \mu\text{m}^3$, respectively). This result indicates that D1 is not a 'division' mutant which is defective in septation or the cell separation step.

Thymidine addition (final concentration of 100 µg ml⁻¹)

Fig. 5. $[^3\text{H}]$ Thymidine incorporation of the stationary-phase cultures of *D. radiodurans* IR ($\circ$, $\bullet$) and D1 ($\square$, $\blacksquare$) in PCB with ($\bullet$, $\blacksquare$) and without ($\circ$, $\square$) Mn(II) addition. Cultures were grown in PCB to enter stationary phase at 32°C . Each culture was made in duplicate. Mn(II) ($10\ \mu\text{M}$) and $[^3\text{H}]$ thymidine ($20\ \mu\text{Ci ml}^{-1}$; $740\ \text{kBq ml}^{-1}$) were simultaneously added to one of duplicate cultures, and $[^3\text{H}]$ thymidine only was added to the other one. Cultures were sampled at short intervals for determining radioactivities. Individual points represent the mean of three independent measurements differing by less than 10%.

to the stationary-phase cultures of IR and D1 in PCB did not induce extra cell division. The incorporation of $[^3\text{H}]$ thymidine was, therefore, used to measure the DNA synthesis after Mn(II) addition to the stationary-phase cultures of Mn-CD-positive strain IR and negative strain D1. When $[^3\text{H}]$ thymidine was added [$20\ \mu\text{Ci ml}^{-1}$ ($740\ \text{kBq ml}^{-1}$)] to the stationary-phase cultures of both strains at 32°C , no significant $[^3\text{H}]$ thymidine incorporation was observed in both cultures (Fig. 5). When Mn(II) ions ($10\ \mu\text{M}$) and $[^3\text{H}]$ thymidine were added simultaneously to those cultures, the incorporation radioactivities of $[^3\text{H}]$ thymidine also could not be detected in D1, but rapid incorporation occurred in IR (Fig. 5). These results indicate that the Mn-CD-negative mutant D1 has lost DNA synthesis capability induced by Mn(II) ions, and suggest that D1 is possibly defective in some step(s) essential for DNA replication in the Mn-CD process, but not in the N-CD process. The mutation(s) in D1 possibly blocks DNA replication, directly or indirectly.

In bacteriophage, DNA replication can be initiated through several different mechanisms (Kornberg, 1980). *E. coli* has been reported to possess an alternative pathway of DNA replication (Niwa *et al.*, 1979, 1981; Asai & Kogoma, 1994). In this study, DNA synthesis of *D. radiodurans* TS1 was 'immediately halted' after raising to 39°C (Fig. 1b); thus, TS1 is presumably defective in either the production of precursors or the DNA-synthesizing machinery itself but not in initiation of DNA

Fig. 6. Transition of growth ($\square$) and DNA synthesis ($\circ$, $\bullet$) of *D. radiodurans* IR grown in PCB supplemented with Mn(II) . IR cells were grown to late-exponential phase in PCB without ($\circ$) and with ($\bullet$) Mn(II) addition ($10\ \mu\text{M}$), and then $[^3\text{H}]$ thymidine ($20\ \mu\text{Ci ml}^{-1}$; $740\ \text{kBq ml}^{-1}$) was added. Radioactivity and c.f.u. measurements were determined by sampling at a short interval. Growth data are means of at least three replications. Individual points of radioactivities represent the mean of three independent measurements differing by less than 10%.

replication (Hirota *et al.*, 1968; von Meyenberg & Hansen, 1987). TS1 could still exhibit the Mn-CD at 39°C ; consequently, we could not rule out the possibility that a different DNA replication pathway may be involved in the Mn-CD process.

There is a transition lag in the N-CD to Mn-CD shift

When *D. radiodurans* IR was grown in PCB containing $10\ \mu\text{M}$ Mn(II) , the maximum cell number was about $2 \times 10^9\ \text{c.f.u. ml}^{-1}$, which was almost equal to that obtained from the growth after Mn(II) addition to its stationary-phase cultures. Obviously, in the former case growth operated through two growth steps, i.e. the normal and the Mn-CD steps. We suppose that a growth transition may occur due to a shift from N-CD to the Mn-CD pathway when IR cells are cultivated in PCB supplemented with Mn(II) . To test this supposition, a DNA synthesis experiment was conducted. IR cells were grown in PCB, with ($10\ \mu\text{M}$) and without Mn(II) addition, to reach late-exponential phase (approximately $5 \times 10^7\ \text{c.f.u. ml}^{-1}$) and then $[^3\text{H}]$ thymidine [$20\ \mu\text{Ci ml}^{-1}$ ($740\ \text{kBq ml}^{-1}$)] was added. By sampling at short intervals to determine the incorporation of $[^3\text{H}]$ thymidine, we noted that there was a slower rate of incorporation for a short period that corresponded to the very early stationary phase of normal growth in PCB without Mn(II) supplementation (Fig. 6). A slight growth lag in that period was also detected in PCB supplemented with Mn(II) (Fig.

Table 1. Comparison between the cells derived from the Mn-CD effect and those from the N-CD process

The cultures of *D. radiodurans* IR were grown in PCB medium with shaking at 32 °C.

Property	Mn-CD	N-CD
Dividing phase	Non-exponential; doubling time increases with time	Exponential phase with constant doubling time
Cell size*	Approx. 4 µm ³	Approx. 9 µm ³
Colony colour	Pale red	Bright red
Sensitivity to salt-mediated multicell formation effect†	No	Yes
Resistance (LD ₉₀)‡		
UV (J m ⁻²)	500	1350
γ-Ray (krad)	420	1300
NTG (µg ml ⁻¹ h ⁻¹)	435	1225
MMC (µg ml ⁻¹ min ⁻¹)	70	215
Specific activity [U (mg proteins) ⁻¹]§		
Superoxide dismutase	94	19
Catalase	1200	710

* Mean volumes of N-CD cells (early-stationary phase) and Mn-CD cells [those at 11 h after Mn(II) addition to an early-stationary-phase culture] were determined with a Coulter counter.

† Refer to Chou & Tan (1991) for this multicell formation effect. NaCl (0.2–1.2%) was added to an exponential-phase culture (N-CD) and a dividing Mn-CD culture [those at 5 h after Mn(II) addition to an early-stationary-phase culture].

‡ LD₉₀ is the dose of radiation which kills 90% of the exposed cells; data were those for early-stationary-phase cells. Refer to Chou & Tan (1990) for the results of UV and γ-ray treatments. Cells were treated with 350 µg NTG ml⁻¹ and 5 µg mitomycin C (MMC) ml⁻¹.

§ Refer to Chou & Tan (1990).

6). This suggests that the incorporation (or growth) lag appears to be the result of a growth transition from N-CD to the Mn-CD stage. This transition lag is reminiscent of a similar lag seen late in liquid culture of *Streptomyces* spp. (Holt *et al.*, 1992; Leskiw *et al.*, 1993) that seems to involve major changes in protein profiles.

Characteristics of the Mn-CD process

The results obtained from this and previous studies show that cells undergoing Mn-CD display profound physiological changes, as compared with cells derived from the N-CD process (Table 1). In *B. subtilis* (Oh & Freese, 1976) and *Lactobacillus plantarum* (Archibald & Fridovich, 1981), Mn(II) supplementation is required for optimal growth. In these cases, Mn(II) deficiency results in a halt in growth at a low cell number, and the exponential growth rate is increased with Mn(II) addition within a dose range. In contrast, in *D. radiodurans* Mn(II) addition to the medium increases the maximum cell numbers, but not the exponential growth rate (Chou & Tan, 1990). Obviously, different mechanisms of maximum growth enhancement appear to be used in these two cases; the Mn-CD process should not be considered as simply an outcome of optimization of growth conditions. The significant difference of physiological response in Mn-CD cells in comparison with N-CD cells indicates that the onset of

the stationary phase, i.e. the shutdown of the N-CD pathway, is not due to Mn(II) deficiency in the growth medium. Mn(II) may be involved in an induction process which initiates an alternative growth pathway; otherwise the physiological properties of Mn-CD cells in Mn(II)-supplemented medium would be similar to those of N-CD cells in control medium.

The Mn-CD effect produces highly modified cells and appears to be *Deinococcus*-specific. It can provide an attractive system for studying the many novel physiological and genetic properties in deinobacteria which are largely not elucidated (Work, 1964; Baumeister *et al.*, 1986; Counsell & Murray, 1986; Chou & Tan, 1991; Murray, 1992). For example, the fact (Table 1) that in the progression of the Mn-CD cycles cells concomitantly lose their resistance to a variety of DNA-damaging agents and increase synthesis of the free radical scavenging enzyme superoxide dismutase should render the Mn-CD effect useful for studying the mechanism of radioresistance in *D. radiodurans*. Recently, Minton (1994), based on extensive genetic studies, suggested that the extremely high radioresistance in deinobacteria be attributed to an effective way of interchromosomal recombination. Being a reductive type of growth, the Mn-CD process possibly produces cells successively lessening in genome multiplicity or failing in synthesis of a factor(s) critical to the extreme radioresistance.

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