Salt-Mediated Multicell Formation in *Deinococcus radiodurans*

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The highly radiation-resistant tetracoccal bacterium *Deinococcus radiodurans* exhibited a reversible multicell-form transition which depended on the NaCl concentration in the medium. In response to 0.8% NaCl addition into the medium, the pair/tetrad (designated 2/4) cells in a young culture grew and divided but did not separate and became 8-, 16-, and 32-cell units successively. In exponential growth phase, the cells divided in a 16/32 pattern. Potassium ions were equally effective as Na\(^+\) in mediating this multicell-formation effect; Mg\(^2+\), Li\(^+\), and Ca\(^2+\) also worked but produced less multiplicity. This effect appears to be species specific. Thin-section micrographs revealed that in a 16/32-cell unit, eight 2/4 cells were encased in an orderly manner within a large peripheral wall, showing five cycles of septation. Our results suggest the presence of a salt-sensitive mechanism for controlling cell separation in *D. radiodurans*.

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*Deinococcus* species are characterized by their extreme radioresistance (1, 17). They also have long been known to have unusual cell wall amino acids (33), polar lipids (8), and surface layers (3). Recently, while studying the growth effects of metal ions on *Deinococcus* (formerly *Micrococcus*) *radiodurans*, we found that Mn(II) induces cell division in aging deinococcal cultures (5) and increases NaCl tolerance (24). In spite of being thick walled and desiccation resistant, *D. radiodurans* is unexpectedly salt sensitive (25, 31). These findings prompted us to further investigate the effects of salt on this bacterium. The addition of 0.8% NaCl in plate count broth (PCB) medium resulted in a growth curve with an unusually long lag phase for *D. radiodurans* IR. During the NaCl-induced lag phase, *D. radiodurans* cells grew and formed septa, but cell separation did not occur. Instead, the cells shifted from the usual pairs/tetrads (designated 2/4) (18) to 4/8, 8/16, and finally 16/32 forms before entering the exponential phase, dividing in a 16/32 separation mode. In this report, we describe and discuss this phenomenon of salt-mediated multicell formation in detail.

(Portions of this work have been presented elsewhere [6].)

MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *D. radiodurans* IR has been characterized previously (5, 25–28). Sources of other cultures and cultivation conditions were as described previously (5). All media except TGY (1) and LB broth (16) were purchased from Difco Laboratories. Unless otherwise specified, PCB was used for preparing broth cultures.

**Determination of cell number, cell mass, and radiation resistance.** These were essentially as described previously (5). A 16/32 multicell as well as a 2/4 cell each formed 1 CFU.

**Chemicals and salt treatments.** Salts and organic compounds, of at least reagent grade, were purchased from E. Merck AG. For a salt treatment, 1 ml of concentrated salt solution in deionized water was added to a 9-ml culture to obtain the desired concentration.

**Cell morphology.** Phase-contrast microscopy was as described previously (5). For scanning electron microscopy, cells pelleted from broth were fixed in 2% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4). After 2 h of fixation at 4°C, a drop of the suspension was placed on a 13-mm filter (0.22-μm pore size; Millipore Corp., New Bedford, Mass.) for 5 min at room temperature. The filter was washed three times with cacodylate buffer and then dehydrated stepwise in 50, 70, 90, 95, and 100% ethanol for 15 min each. The filter with adherent cells was then critical point dried in liquid CO\(_2\) in a DCP-1 critical point drying apparatus (Denton High Vacuum, Cherry Hill, N.J.), mounted on a stub, and coated with gold to a thickness of 5 to 10 nm. Specimens were examined and photographed with a Hitachi H300 electron microscope with an H3010 scanning attachment (Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV.

**Thin-section microscopy.** The procedures for embedding and sectioning were those outlined by Murray et al. (18) with some modifications. The cells were pelleted from the broth and resuspended in the fixative. The fixation procedures were as follows: suspension in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 2 h at room temperature (25°C), then a wash in buffer, followed by 2 h in 1% OsO\(_4\), and then a wash in buffer. Sections were cut from fixed cells embedded in Epon 812 resin after dehydration in an ethanol series. Reichert-Jung Ultracut E was used to obtain sections showing silver-gray interference colors. Conventional staining of sections was obtained with lead citrate and uranyl acetate according to the method of Reynolds (20). Electron micrographs were taken on Philips electron microscope EM-300 at 60 kV and recorded on Kodak fine-grain-positive 36-mm films.

RESULTS

**Effect of NaCl on the growth of *D. radiodurans* IR.** NaCl was added into a PCB culture to a final concentration of 0.8% (138 mM), and the growth curve of treated cells was compared with that of the untreated culture. As shown in Fig. 1A, a slower exponential growth rate and a 7-h lag phase were exhibited by the salt-treated culture. However, an increase in culture turbidity occurred during this 7-h lag phase. Phase-contrast and scanning electron micrographs (Fig. 1B) revealed that in response to added NaCl, IR cells shifted from the typical 2/4 form to 4/8, 8/16, and 16/32 units. Thus, cells actually divided but did not separate during the lag phase. Cells then separated in the 16/32 form during the exponential phase and remained in this form while in stationary phase.

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Cell growth and division are necessary for this salt-mediated multicell formation. When 0.8% NaCl was added to cultures at transition from exponential to stationary phases, cells that entered the stationary phase were in the 4/8 or 8/16 form, depending on the time that NaCl was added (data not shown). The later NaCl was added, the less cell multiplicity was found. No multicell forms were observed after addition of NaCl to stationary-phase cultures.

Dose dependence of the multicell-formation effect. The NaCl-induced multicell formation was concentration dependent up to partially inhibitory concentrations (Table 1). The addition of as little as 0.4% NaCl triggered this effect. At NaCl concentrations up to 0.6%, the maximum growth was not affected. The decrease in CFU per milliliter was simply a manifestation of the change from the 2/4 to the 4/8 or 8/16 form. However, even at 0.4% NaCl, the doubling time during the exponential phase increased. At NaCl concentrations of 1.2% or higher, cell arrangement was in a much less orderly manner, and groups varied in size (results not shown).

Specificity of multicell formation. Various salts were tested for their ability to trigger the multicell formation. Salt concentrations which ranged from noninhibitory to partially inhibitory to inhibitory were used (Table 2). All tested sodium and potassium salts stimulated multicell formation. Moreover, Na$^+$ and K$^+$ (on an equimolar basis) were identical in triggering the effect (data not shown). Therefore, the cation plays the major role in the multicell-formation

### Table 1. Changes in growth parameters as a result of NaCl addition

<table>
<thead>
<tr>
<th>NaCl addition (%)</th>
<th>Exponential growth</th>
<th>Maximum growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Doubling time (min)</td>
<td>Mode of cell separation</td>
</tr>
<tr>
<td>0</td>
<td>67</td>
<td>2/4</td>
</tr>
<tr>
<td>0.4</td>
<td>75</td>
<td>4/8</td>
</tr>
<tr>
<td>0.6</td>
<td>90</td>
<td>8/16</td>
</tr>
<tr>
<td>0.8</td>
<td>110</td>
<td>16/32</td>
</tr>
<tr>
<td>1.0</td>
<td>125</td>
<td>16/32</td>
</tr>
</tbody>
</table>

*a* To PCB medium.  
*b* Dry weight.
The tetracoccal bacteria *Micrococcus roseus* and *Micrococcus luteus* had no NaCl-stimulated multicell formation. Moreover, other strains of *Deinococcus radiophilus*, *Deinococcus proteolyticus*, and *Deinococcus radiognanas* also tested negative. However, *D. radiodurans* R1 (the type strain) had an essentially identical multicell-formation response as did strain IR. Therefore, the effect appears to be species specific.

**Thin-section micrographs of 2/4 and 16/32 cells.** To characterize the cell septation and separation patterns of the cells, ultrathin-section micrographs which showed the fine structures of the 16/32 and 2/4 cells were compared (Fig. 2). Septation and separation of 2/4 cells of strain IR (Fig. 2A) patterned similarly to strain R1 of *D. radiodurans*, which is commonly cultivated in low-salt media and has been extensively studied for fine-structure analysis (22), particularly of the cell wall architecture (3). Like the 2/4 cells, the 16/32 cells divide alternately in two planes at right angles, and five cycles of division within a single CPW lead to the formation of a bacterial sheet which is only one cell thick (Fig. 2B and C). It should be pointed out that since the 16/32 cells are only one cell thick but four cells wide and eight cells long, the chance that the sectioning plane is parallel to the bacterial sheet is considerably low; several hundred multicells need to be searched for the one shown in Fig. 2B. The 16/32 cells were similar to the 2/4 cells coenclosed in a peripheral wall (PW). The sizes of the two PWs are different, but the layered structure and thickness appear similar. In both 2/4 and 16/32 cells, septation begins as a small ingrowth of wall, and plasma membrane at the periphery is progressed toward the center (cross wall [CW] in Fig. 2A; CW4 in Fig. 2B). The time order of formation can be easily assigned to the various septa in a 16/32 cell (Fig. 2B, in order of increasing maturity: CW4, CW3, CW2, CW1, SW). SW indicates a mature septum ready for or undergoing separation. It is noted in Fig. 2B and E that CW1 and CW2 are essentially identical in thickness, whereas CW3 is thinner, suggesting that a step after the completion of the septum is retarded as a result of the salt treatment. The overall picture of the 16/32 cells suggested that the multicells were basically a group of 2/4 cells and that the individual 2/4 cells divided in certain degrees of synchrony.

**Radiation resistance of 16/32 cells.** As shown in Fig. 3, the 16/32 cells showed slightly higher resistance to the killing effects of both UV and γ-ray radiation than did the 2/4 cells. After the irradiation treatment, the 16/32 cells remained in their multicell form before being plated for survivor enumeration. Since the survival was expressed as CFU, the increased radioresistance was probably due to the fact that a 16/32 unit contains more cells than a 2/4 unit.

**An extracellular heat-labile factor in multicell formation.** Results shown in Fig. 2 suggest that multicell formation relates to a key step in cell separation, which is partially inhibited in the multicells. In bacteria, there is evidence for a role of a lytic enzyme in septum separation (14, 23, 32).

**Effect of individual salts, at least four concentrations in this range were tested, including those which partially inhibited the growth of the culture.**

<table>
<thead>
<tr>
<th>Salt</th>
<th>Range of concen tested (mM)</th>
<th>Cell multiplicitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>35–175</td>
<td>16/32</td>
</tr>
<tr>
<td>NaI</td>
<td>15–50</td>
<td>8/16</td>
</tr>
<tr>
<td>NaF</td>
<td>15–50</td>
<td>8/16</td>
</tr>
<tr>
<td>Na2SO4</td>
<td>30–75</td>
<td>8/16</td>
</tr>
<tr>
<td>Sodium glutamate</td>
<td>35–175</td>
<td>16/32</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>15–40</td>
<td>8/16</td>
</tr>
<tr>
<td>KCl</td>
<td>35–175</td>
<td>16/32</td>
</tr>
<tr>
<td>KI</td>
<td>35–100</td>
<td>8/16</td>
</tr>
<tr>
<td>KF</td>
<td>20–40</td>
<td>8/16</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>35–60</td>
<td>8/16</td>
</tr>
<tr>
<td>LiCl</td>
<td>35–60</td>
<td>4/8</td>
</tr>
<tr>
<td>CsCl</td>
<td>1–2</td>
<td>2/4</td>
</tr>
<tr>
<td>MgCl2</td>
<td>1.5–50</td>
<td>8/16</td>
</tr>
<tr>
<td>MgSO4</td>
<td>1.5–10</td>
<td>4/8</td>
</tr>
<tr>
<td>CuCl2</td>
<td>5–15</td>
<td>4/8</td>
</tr>
<tr>
<td>CoCl2</td>
<td>0.01–1.5</td>
<td>2/4</td>
</tr>
<tr>
<td>CuCl2·2H2O</td>
<td>0.01–0.05</td>
<td>2/4</td>
</tr>
<tr>
<td>ZnCl2</td>
<td>1.5–10</td>
<td>2/4</td>
</tr>
<tr>
<td>FeCl3</td>
<td>1.5–10</td>
<td>2/4</td>
</tr>
<tr>
<td>MnCl2</td>
<td>0.8–1.5</td>
<td>2/4</td>
</tr>
</tbody>
</table>

- a For individual salts, at least four concentrations in this range were tested, including those which partially inhibited the growth of the culture.
- b The numbers denote the highest cell multiplicity obtained. 2/4 indicates no change, i.e., salt-treated cells remained growing in pairs and tetrads.
- c Cells were disordered in their arrangement.

**Fig. 2.** Thin-section micrographs of 2/4 and 16/32 cells of *D. radiodurans* IR. (A) Typical 2/4 cell with a mature CW (the separation wall [SW]), two incomplete septa, and a PW surrounding the 2/4 cell. (B) 16/32 cell which shows eight 2/4 cells surrounded by a PW. CW1 and CW2, inter-2/4 CWs; CW3, intra-2/4 cross wall; CW4, developing septum. (C) Thin section showing that cells in a 16/32 unit are composed of a sheet which is one cell thick. The rectangular box shows a 16/32 unit sectioned in a plane perpendicular to the sheet of bacteria. (D) Close-up of the central part of Fig. 2B. Arrow indicates PW. R, Unidentified residual material. (E) Close-up of a diamond-shaped rarefaction in Fig. 2B where CW1 and CW2 meet, showing that the CW1 and CW2 appear to be identical in thickness (i.e., maturity) and that the separation was already initiated. Each bar = 1 μm.
MULTICELL FORMATION IN D. RADIODURANS
The enzyme may be associated with the outer membrane, and the binding appears to be loose (11, 30). To test whether the multicell-formation effect is mediated through an inhibition of the synthesis or the activity (or both) of a similar enzyme system, the supernatant of a PCB-grown exponential 2/4 culture, which might serve as a source of the enzyme(s), was used to suspend pelleted 16/32 cells. The cell suspension was incubated as usual, and cell samples were taken at intervals for microscopic observation (Fig. 4). Shortly after the treatment started, the cell envelope of the 16/32 cells appeared to be loosened (Fig. 4B). Later, the 16/32 cells were found falling apart, resulting in the formation of packages of 8 or 16 cells (Fig. 4C). Also observed was that 2/4 supernatant with NaCl added at 0.8% had the same effect on the 16/32 cells. Given continued cultivation, a supernatant-treated culture eventually divided in a 2/4 mode, whereas a supernatant-plus-salt culture resumed the 16/32 pattern. Presumably, in the latter case, the turnover of the effective factor(s) in the suspending medium allowed the added NaCl to exert the multicell-formation effect. If pelleted 16/32 cells were resuspended and incubated in fresh PCB medium, more time was needed for the initiation of cell separation (ca. 55 min versus 15 min). These results showed that the supernatant of the 2/4 culture might contain a lytic
enzyme responsible for cell separation, the activity of which was not inactivated by NaCl. Therefore, NaCl probably inhibits its synthesis or maturation. This activity appeared to involve a heat-labile factor, since boiled (5 min) supernatant of 2/4 culture was not effective (data not shown).

DISCUSSION

In this paper, we report that low concentrations of certain salts in the growth medium induce dramatic changes in the appearance of *D. radiodurans*. These cells are normally found as either pairs or tetrads during the exponential phase of growth. When cells are grown in the presence of 0.8% NaCl, however, cell division is altered and cells are found in regular arrays of either 16 or 32 cells. This multicell effect presents a novel growth-related salt response which is characterized by its specific action on cell separation. The time interval between completion of cell septation and separation was prolonged. The rate of cell separation decreased with increases in the NaCl concentration. It is possible that *D. radiodurans* cells respond to the increased salt concentration in the environment with a new balance in the two key processes of cell division, namely septation and separation (21). The 4/8, 8/16, or 16/32 forms of cell division are each individually new balances of cell separation and septation. The presence and stability of multicells require balanced growth among the PW-enclosed individual 2/4 cells. As the salt concentration approaches inhibitory levels, cells in the same PW lose synchrony. Only at a concentration relatively higher than the inhibitory concentration of NaCl was the balance between growth and cell division altered to such an extent that cell morphology became abnormal and cell growth was inhibited. These results at least partly explain the unexpectedly high level of salt sensitivity of *D. radiodurans*, which is thick walled (17) and drying resistant (25, 31). There is limited information on the mechanisms of cell separation in *Escherichia coli* (23, 32) and streptococci (13, 14). Our findings of the multicell effect should provide a convenient system, in which cell separation and septation are dissociated from each other, for studying the mechanisms of cell separation. The multicell effect thus provides evidence that cell separation is a distinct part of the total process of cell division compared with the stages leading to septum formation (7).

We demonstrated that this multicell phenomenon is readily reversible by resuspending NaCl-treated cells in culture media taken from exponential-phase cultures of *D. radiodurans* grown in the absence of NaCl. We established that this reversal is the result of a heat-labile factor released by growing cells into the growth medium. Our data suggest that the effect is mediated by partially inhibiting the synthesis or maturation and activation of a cell-separating enzyme rather than directly inhibiting the mature enzyme. This effect in *D. radiodurans* is apparently not caused by modification of the cell wall structures.

The salt-mediated multicell effect is dose dependent and can be reversed by inoculating the multilocs into the low-NaCl media. Our results show that both growth rate reduction and cell osmoregulation appear not to be the cause of multicell formation. Moreover, multicell formation can be caused by five different cations. Thus, the intracellular ionic strength which may specifically modulate gene expression (10) may have some importance. Certain modifications in bacterial cell morphology such as size (2) and shape (15, 29) have been reported to correlate with the ionic content of cytoplasm.

As with many other similar physiological (5, 25) and biochemical (28) properties shared between *D. radiodurans* IR and R1, the multicell effect was also found reproduced in R1. Since strains of three other known *Deinococcus* species did not produce multicell forms, it appears to be specific to *D. radiodurans* and does not seem to be related to the extreme radioresistance in deinococcal bacteria. The multicell effect causes no loss of radioresistance, suggesting that any proteins that are synthesized in response to radiation damage or whose activity is necessary for damage repair are unaffected by NaCl treatment. One other genus-specific property of *Deinococcus* that we have reported recently is the Mn(II)-induced cell division (5). These two properties are easy to demonstrate and may provide useful distinguishing features for identification of the species *D. radiodurans*.

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