Killed cultures of *Escherichia coli* can protect living organisms from acid stress

Polluting and contaminating enterobacteria, often face lethal chemical or physical agents or conditions, with exposures to acidity, alkalinity, metal ions, heat or antibiotics, for example, being common. Provided that organisms in cultures exposed to such lethal conditions were dead and not simply moribund, the exposed cultures would not be expected to influence the behaviour of living organisms which entered the environment later, except perhaps by providing nutrients. This report shows, however, that dead cultures can confer an acid stress tolerance legacy on living organisms.

Acid tolerance is induced rapidly when enterobacteria, growing at neutral pH, are transferred to mildly acidic external pH (1, 4). It has been assumed that external acidity is detected by an intracellular sensor whose activation triggers a series of intracellular reactions (1). In common with other stress responses (2, 3), however, recent work suggests that tolerance of *Escherichia coli* to potentially lethal external acidity is absolutely dependent on secretion into the medium of an extracellular induction component (EIC), needed for acid tolerance induction at pH 5.0, which also induces tolerance when added in neutralized filtrates to organisms growing at pH 7.0 (4). This EIC arises on activation, by mild acidity, of an extracellular sensing component (ESC) produced at neutral or acidic pH, activation occurring in the absence of organisms (5). The acid-stress-related ESC and EIC are not irreversibly inactivated by high stress levels (6), and so it seemed possible that killed cultures might contain these components and induce tolerance. This proved to be so; some dead *E. coli* cultures induced acid tolerance in living organisms cultured at pH 7.0 (Table 1).

Acid tolerance is conferred either by filtrates from pH 7.0-grown organisms, activated at pH 5.0, or by filtrates from pH 5.0-grown cultures, and so three types of killed culture were used, namely (a) killed pH 7.0-grown cultures without activation, (b) killed pH 7.0-grown cultures activated at pH 5.0 after killing and neutralized before use, and (c) cultures grown at pH 5.0, neutralized and killed. All killing methods employed (Table 1) produced almost full lethality, the highest percentage survival being 2% after nobileacin (novo) treatment. Three killing methods (70 °C, 15 min; pH 11.0, 15 min; pH 2.0, 15 min) were used on all three types of culture, but novo (5 µg ml⁻¹, 120 min) was used only at pH 5.0, as at pH 7.0, > 100 µg novo ml⁻¹ is needed for a 95% kill. Finally, Cu²⁺ (30 µg ml⁻¹ for 60 min at 37 °C) was used at pH 7.0 or 5.5 for killing, with activation at pH 5.5.

Table 1 shows that 99% of exponential-phase pH 7.0-grown organisms were killed in 7 min at pH 5.0. Such organisms, however, became acid-tolerant if pre-incubated with certain killed cultures. First, heat-killed cultures showed tolerance-inducing effects on living organisms pre-incubated with them at pH 7.0 (Table 1). Heat-killed pH 7.0 culture not activated at pH 5.0 had a small effect, possibly due to partial ESC activation at 70 °C (6). The acid-tolerance-inducing effect was markedly increased for heat-killed pH 7.0-grown culture subsequently activated at pH 5.0, with 394 ± 2.2% of preincubated organisms surviving acid. Killed pH 5.0-grown culture had a smaller effect, but conferred increased tolerance (Table 1). Similar acid-tolerance-inducing effects were obtained with the other killed cultures (Table 1).

It is assumed that the above effects resulted from the presence of EICs and ESCs in killed cultures. In accord with this, protease treatment of killed cultures, which destroys EICs and ESCs in medium (4, 5, 6), abolished tolerance-inducing ability, whilst dialysis (acid tolerance ESC and EIC are nondialysable) did not prevent killed cultures from inducing acid tolerance.

It is probable that killed cultures induce specific acid tolerance. An alternative is that killed cultures reduce growth rates of pH 7.0-grown cultures. Slowly growing cells are more acid-tolerant than rapidly growing ones, but killed cultures had no effect on growth rates. Another explanation is that killed cultures alter the pH of cultures during incubation, but the pH remained at 7.0 ± 0.1 throughout tolerance induction.

In most cases, less than 0.1% of organisms survived the killing treatments (Table 1), and so acid-tolerant organisms surviving after incubation and acid challenge could not have been survivors from the killed cultures. However, in case some of the ‘dead’ organisms were merely moribund and recovered during incubation with living culture, two further tests were undertaken. First, incubation of
**Table 1.** Killed cultures can induce acid tolerance in living organisms

Strain 1829 ColV was grown in broth to exponential phase at the stated pH and, after neutralization if necessary, cultures were killed as described in the text. Viable counts were undertaken on killed and untreated cultures, plating samples on nutrient agar. Percentage survival values are shown for representative experiments. NA, Not applicable.

<table>
<thead>
<tr>
<th>Killed culture used for acid tolerance induction</th>
<th>Viable cells remaining in killed culture (%)</th>
<th>Activation of killed culture</th>
<th>Percentage survival after acid treatment for pH 7.0-grown cells pre-incubated with or without killed culture*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>1.0 ± 0.15</td>
</tr>
<tr>
<td>pH 7.0 culture, heat-killed</td>
<td>0.007</td>
<td>None</td>
<td>6.9 ± 1.2</td>
</tr>
<tr>
<td>pH 7.0 culture, heat-killed</td>
<td>0.007</td>
<td>At pH 5.0</td>
<td>39.4 ± 2.2</td>
</tr>
<tr>
<td>pH 5.0 culture, heat-killed</td>
<td>0.0001</td>
<td>None</td>
<td>10.9 ± 0.4</td>
</tr>
<tr>
<td>pH 7.0 culture, alkali-killed</td>
<td>0</td>
<td>None</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>pH 7.0 culture, alkali-killed</td>
<td>0</td>
<td>At pH 5.0</td>
<td>11.8 ± 0.2</td>
</tr>
<tr>
<td>pH 5.0 culture, alkali-killed</td>
<td>0.0002</td>
<td>None</td>
<td>13.3 ± 0.2</td>
</tr>
<tr>
<td>pH 7.0 culture, acid-killed</td>
<td>0.0001</td>
<td>None</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>pH 7.0 culture, acid-killed</td>
<td>0.0001</td>
<td>At pH 5.0</td>
<td>17.5 ± 1.1</td>
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<tr>
<td>pH 5.0 culture, acid-killed</td>
<td>0.6†</td>
<td>None</td>
<td>16.6 ± 1.2</td>
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<td>pH 5.0 culture, novo-killed</td>
<td>2.0</td>
<td>None</td>
<td>31.9 ± 0.3</td>
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<td>pH 7.0 culture, Cu²⁺-killed</td>
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<td>None</td>
<td>8.2 ± 0.4</td>
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<tr>
<td>pH 7.0 culture, Cu²⁺-killed</td>
<td>0.02</td>
<td>At pH 5.5</td>
<td>14.1 ± 0.4</td>
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<tr>
<td>pH 5.5 culture, Cu²⁺-killed</td>
<td>0.09</td>
<td>None</td>
<td>38.5 ± 0.5</td>
</tr>
</tbody>
</table>

* Killed cultures (neutralized if necessary) were incubated with exponential-phase pH 7.0 broth-grown cultures of 1829 ColV (1 part killed culture to 1 part exponential-phase culture, numbers of killed and living organisms approximately the same in mixtures) for 45 min at pH 7.0. Mixtures were washed with pH 7.0 broth and challenged at pH 3.0 for 7 min before plating on nutrient agar with incubation at 37°C. Results are expressed as percentage survival ± SEM after acid challenge for three replicates.

† Increased survival was observed for acid-killed pH 5.0-grown cultures, compared to pH 7.0-grown cultures; such cultures have induced acid tolerance, which partially protects them from pH 2.0.

killed culture with fresh broth did not allow organisms to recover and form colonies; the slightly increased numbers plating in this situation derived from division of the few live organisms. Second, to prove conclusively that acid-tolerant organisms present, after incubating living *E. coli* with killed cultures, did not derive from survivors in the killed culture, the following test was undertaken. A pH 5.0-grown streptomycin-resistant culture was neutralized, killed with alkali and used to induce tolerance in a streptomycin-sensitive one. All of the organisms surviving acid challenge were streptomycin-sensitive, i.e. they did not derive from the killed streptomycin-resistant culture.

These results mean that even if contaminating organisms have been killed, the killed culture may still contain agents which confer stress tolerance on organisms subsequently entering the environment. These findings are likely to have relevance for the effects of polluting organisms in natural waters. If they were killed by Cu²⁺, for example, stress tolerance could later be conferred on enterobacteria which subsequently entered the body of water. Also, if stress-tolerant organisms in food were killed by heating, for example, if the food were later ingested with other contaminated food or water, stress tolerance could be conferred by the killed culture on the contaminating organisms. It is likely that appropriate killed cultures can induce other stress responses in living organisms. I have shown that alkali tolerance and alkali sensitivity can be conferred by suitable killed cultures.

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On the zymogenic character of chitin synthase 3

The paper by Ono et al. (3) contains some statements that amount to a misconception of our previous results (2). In that study, we showed that chitin synthase 3 (*chs3*) activity in a membrane preparation was not activated by trypsin, but became zymogenic (activated by protease) after extraction of the membranes with detergents. In *cafl* (now called *chs4*) mutants, the Chs3p activity in the membrane preparation was zymogenic without any detergent extraction. Based on our results and Bulawa’s finding (1), that overexpression of *CHS4* led to an increase in Chs3p activity, we proposed that Chs3p was non-zymogenic in wild-type membranes because it was associated with an activator, Chs4p. Removal of Chs4p by detergents or by mutation would leave Chs3p in the zymogenic state. The enzyme could then be activated by an alternative route, namely partial proteolytic digestion.

Ono et al., like Bulawa, overexpressed *CHS4* and found that Chs3p activity increased. They extracted membranes with a detergent mixture that solubilized, at least in part, Chs4p and Chs3p, and found that the extracted activity was not zymogenic. They comment that, “According to Choi et al. (1994) non-zymogenic Chs3p activity can be released from membranes with detergents, such as CHAPS, though such detergent-extractable Chs3p activity has never been confirmed by others’.

These comments are a misrepresentation.
of our results and conclusions. As mentioned above, we never said that ‘non-zymogenic Chs3p activity can be released from membranes with detergents’. Our interpretation of the results was that Chs4p had been extracted by the detergent, leaving behind Chs3p, which now showed its zymogenic character, previously masked by the presence of the activator. The additional remark that our results have ‘never been confirmed by others’ seems to suggest that others tried and could not confirm them. In fact, I am not aware of any such attempt and failure. Rather, Trilla et al. (1997) confirmed that the Chs3p activity becomes zymogenic when Chs4p is eliminated by mutation, as we had previously found.

Furthermore, in the Discussion, Ono et al. say, ‘As mentioned before, Choi et al. (1994) demonstrated that a certain level of Chs3p activity in membranes is attributable to the zymogenic activity of Chs3p, and that such zymogenic activity is not extracted by detergents. The ratio and physiological significance of zymogenic and non-zymogenic Chs3p activities, however, remain elusive’. This is not at all what we said in our paper. We proposed that all of the Chs3p is zymogenic in the absence of Chs4p, as shown by the chs4Δ mutants (and confirmed by Trilla et al.). Ono et al. only worked in the presence of excess (overexpressed) Chs4p; under these conditions Chs3p is maximally active and cannot be further activated by trypsin. In fact, I do not see any contradiction between their results and ours.

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Authors’ reply

In the paper by Ono et al. (2), we reported that overexpression of CHS4 increased Chs3p activity and that about half of the Chs3p activity in the membranes of yeast over-expressing CHS4 was extracted with CHAPS and cholesteryl hemisuccinate. Furthermore, Chs3p activity extracted with the detergent was non-zymogenic. In this context, we cited the paper by Choi et al. (1), ‘According to Choi et al. (1994) non-zymogenic Chs3p activity can be released from membranes with detergents, such as CHAPS, though such detergent-extractable Chs3p activity has never been confirmed by others’. The authors of the above paper suggest that in this sentence, their work was misrepresented. As shown in Figs 1 and 2 of the paper by Choi et al., the detergent treatment of the membranes caused a decrease in Chs3p activity and they proposed that this decrease after detergent treatment was presumably due to the release of Chs4p. This hypothesis was further supported by the finding that Chs3p became zymogenic even without detergent treatment in the absence of Chs4p. They also found that the increase in Chs3p activity caused by trypsin treatment in the membranes from the cal2 mutant was the same as that observed with the CHAPS-treated membranes from ECY36-3C. Nevertheless, it was not clearly demonstrated whether all of the Chs3p remained in the membranes even after detergent treatment, or whether the detergent released some of the Chs3p activity from the membranes. Since we found that Chs3p activity was released from the membranes by detergent, we suggested that when interpreting the data in Figs 1 and 2 of their paper it is possible that some of the Chs3p activity might be extracted from the membranes. However, we concede that this was our over-interpretation of their results and that the paper by Choi et al. was misrepresented here. We may also have given readers the impression that attempts to identify such activity did not succeed. We simply meant that such activity has not been demonstrated and did not mean to imply that it could not be shown. Therefore, ‘such detergent-extractable Chs3p activity remains unexplored’ would be more appropriate as a conclusion.

As for the criticism regarding the following, ‘As mentioned before, Choi et al. (1994) demonstrated that a certain level of Chs3p activity in membranes is attributable to the zymogenic activity of Chs3p, and that such zymogenic activity is not extracted by detergents. The ratio and physiological significance of zymogenic and non-zymogenic Chs3p activities, however, remain elusive’, Choi et al. demonstrated that the detergent treatment decreased Chs3p activity, which was restored by protease treatment, and that the zymogenic feature of Chs3p became obvious even without detergent treatment in the absence of Chs4p. Furthermore, the increase in Chs3p activity caused by trypsin treatment of the cal2 membranes was the same as that observed in detergent-extracted membranes of ECY-36C. In the letter above, the author mentions that Choi et al. proposed that all the Chs3p is zymogenic in the absence of Chs4p. However, it was not clear as to what percentage of Chs3p was indeed activated by protease treatment in the presence or absence of Chs4p. In fact, Fig. 5 of Choi et al. shows that membranes from the cal2 mutant displayed a certain level of Chs3p activity even without trypsin treatment. However, to avoid any confusion, the sentence starting, ‘As mentioned before, Choi et al. (1994) demonstrated that …’ would have been more appropriately written as, ‘According to the results obtained in this study …’.

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