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Recovery of exponentially growing cultures of *Klebsiella pneumoniae* NCIB 418 after heat shocks

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Abstract. Exponentially growing cultures of *Klebsiella pneumoniae* were subjected to heat shocks in the superoptimal and supermaximal temperature ranges for growth on glucose in a defined mineral salts medium. Transitory changes in the specific growth rate constant during recovery were evident. The response was heat shock temperature and exposure time dependent. Cell viability determinations, based on colony counts, indicated complete recovery from heat treatments at superoptimal temperatures. In contrast, at supermaximal temperatures, discrepancies in colony counts on different agars were observed. The kinetic response of the specific growth rate constant after a heat shock at supermaximal temperatures is explained by segregation within the bacterial population

Key words: Heat shock – Superoptimal/supermaximal temperature – Recovery – Specific growth rate constant – *Klebsiella pneumoniae* – Population – Injury

The development of a better understanding of the response of bacteria to short term heat shocks at temperatures above their optimum for growth and product formation has recently gained in importance, because temperature inducible expression systems for the controlled expression of genes in recombinant bacteria are finding increasing application as alternatives to certain metabolite induced expression systems (Anderson Da Silva and Bailey 1989). One such system involves the temperature-sensitive λ repressor cI857 with either a λ P_L or P_R-promoter, where, to obtain high degrees of expression, bacteria have to be subjected to a temperature shift to and maintenance at 42°C (Lastick et al. 1986). Another system consists of a lac promoter carried on an attnutL-p-att-N gene block which inverts when the bacteria into which this has been introduced are subjected to 42°C for 10 min, thereby causing expression of the combined gene (Podhajska et al. 1985). The latter heat shocking could be accomplished by passage of the bacteria through a heat exchanger and subsequent return to a favourable growth temperature, as opposed to prolonged retention of the bacteria at a superoptimal temperature, that, clearly, is potentially injurious to them. The purpose of the present work was to study the effects of short term heat shocks on the growth kinetics of a bacterial population during subsequent recovery.

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Methods

Organism and cultivation conditions

Klebsiella pneumoniae NCIB 418 was grown in batch culture in a bioreactor (Bioengineering AG, Wald, Switzerland) with an operating volume of 1.4 l. The temperature and the impeller speed were controlled at 35°C and 800 r.p.m., respectively. The pH was maintained constant at 6.8 by automatic addition of either an equimolar 2 M NaOH/KOH mixture or a 10% (w/w) solution of H_3PO_4. The aeration rate was 35 l h^{-1}. The growth medium was a defined mineral salts medium (Evans et al. 1970) modified by replacing citric acid with 55 mg l^{-1} Na_2EDTA, whilst 2.5 g l^{-1} glucose served as the sole carbon energy source. The medium used for the heat treatment and recovery experiments contained 1 g l^{-1} glucose and was buffered with a Na_2HPO_4/KH_2PO_4 mixture (0.1 M for PO_4^{3-}) at pH 6.8.

Heat treatment: Cells were removed from the bioreactor at a predetermined optical density of 1.8, measured at 546 nm, i.e., during the mid-exponential growth phase. Aliquots of 2 ml were immediately transferred into preheated stirred flasks of 100 ml total volume containing 20 ml of culture medium in which the glucose concentration was identical with that in the bioreactor at the time of inoculum removal. The temperatures used for heat shocking were 42°C, 45°C, 46.5°C, 48°C and 51°C. Exposure times were 1, 3 or 5 min. For each series of experiments a control was processed at 35°C. For recovery, the whole content of each flask was transferred into a second stirred 500 ml flask containing 180 ml of the same medium maintained at 35°C.

Growth measurement: Samples were removed at either 6 or 12 min intervals and growth was measured as the increase in the absorbance at 546 nm (OD) using an Uvikon 860 spectrophotometer (Kontron AG, Zürich, Switzerland). The apparent specific growth rate constant (μ) at $t_n = 0.5 (t_2 + t_1)$ was calculated according to the equation:

$$\mu(t_n) = [OD(t_2) - OD(t_1)]/[0.5(OD(t_1) + OD(t_2))(t_2 - t_1)] \quad (1)$$

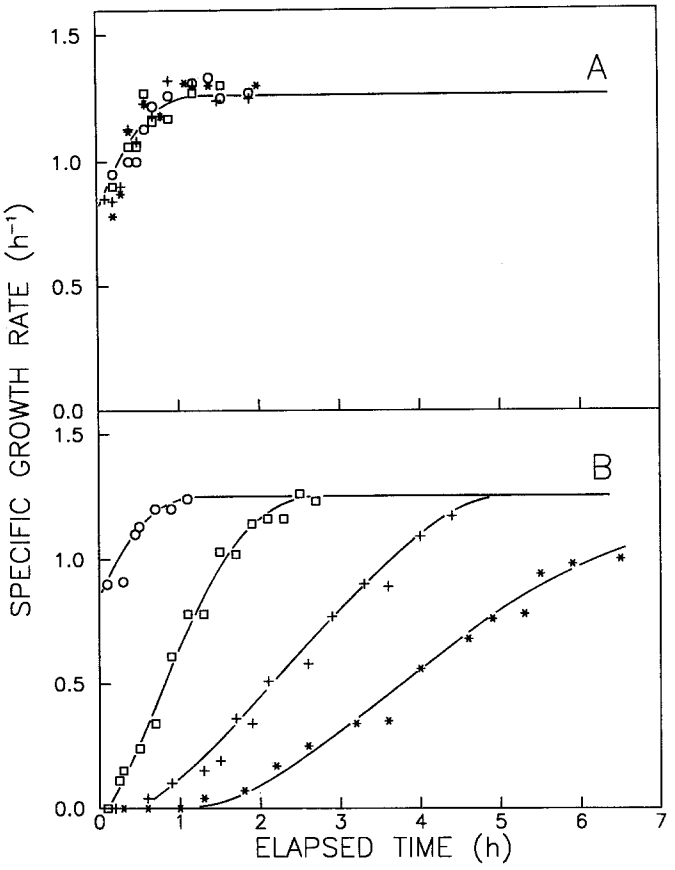

Fig. 1 A, B. Time course of the specific growth rate during recovery of *K. pneumoniae* cultures after heat shocks at 42° C (**A**) and 48° C (**B**). Exposure times were 0 (○), 1 (□) 3 (+) and 5 (*) min. The initial specific growth rate constants of the cultures before the heat treaments were 1.23 h^{-1} (**A**) and 1.20 h^{-1} (**B**)

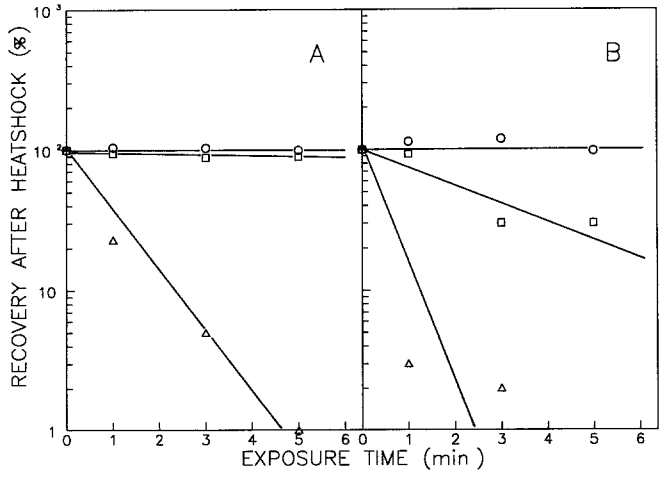

Fig. 2 A, B. Survival of *K. pneumoniae* after heat shocks at 45° C (○), 48° C (□), and 51° C (△) on two different agars: (**A**) plate count agar, (**B**) glucose agar

where t is the time of sample removal in hours and the subscripts 1 and 2 represent initial and final conditions.
All the experiments were carried out either in duplicate or in triplicate and data points represent mean values.

Plate counts: PCA plates are prepared with 2.35 g l^{-1} plate count agar (Difco, Detroit, USA) and 13.5 g l^{-1} Bacto agar (Difco, Detroit, USA) supplemented with 1 g l^{-1} glucose and buffered at pH 6.8 with Na_2HPO_4/KH_2PO_4 (0.05 M for PO_4^{3-}). BG plates were prepared by addition of 15 g l^{-1} Bacto Agar to the mineral medium used for growth experiments, supplemented with 2 g l^{-1} glucose and buffered at pH 6.8. After sample dilution and plating, colonies were counted after incubation at 35° C for 48 h. The counts were carried out in quintuplicate. The results presented are mean values.

Table 1. Recovery times for *K. pneumoniae* cultures subjected to several heat shocks for different exposure times; for definition of recovery time see text

Heat shock temperature (° C)	Recovery time (h)		
	Exposure time (min)		
	1	2	5
42	<0.2	<0.2	<0.2
45	0.2	0.4	0.5
46.5	0.4	0.8	1.3
48	1.3	3.3	5.8
51	5.5	18.3	>18.3

Results

Exponentially growing cultures of *K. pneumoniae* were subjected to heat shocks at either superoptimal (42° C, 45° C) or supermaximal (46.5° C, 48° C, 51° C) temperatures, for the growth of the bacterium on glucose as its sole carbon energy substrate. Response patterns were temperature and exposure time dependent as shown in the results for 42° C and 48° C in Fig. 1. Superoptimal heat shocks were either insignificant (42° C) or resulted in transitory, slightly exposure time dependent, decrease of the specific growth rate constant (45° C). In Fig. 1 B the response of *K. pneumoniae* to a heat shock which is clearly in the supermaximal region (48° C) is shown. An initial lag phase where no growth occurred, was followed by an acceleration phase with respect to the growth rate constant. The recovery times exhibited by *K. pneumoniae* to attain the initial specific growth rate constant of a control, after the several heat treatments, are given in Table 1. Recovery time was defined as the time required for a heat shocked culture to attain the initial growth rate constant of the control.

In Fig. 2 the survival patterns of *K. pneumoniae* plated both on plate count agar and on a minimal glucose agar after different heat shocks are shown. Heat treatments at 45° C for all exposure times investigated allowed complete recovery on both agars. In contrast, discrepancies in colony counts occurred at 48° C. Whilst heat treatment for 5 min allowed essentially complete recovery on plate count agar, an exposure time dependent reduction in survival was observed on minimal glucose agar. At 51° C a reduction in survival on both agars was observed, but was most pronounced on the minimal glucose agar. Figure 3 shows the results of a calculation for the prediction of recovery from a 5 min heat shock at 48° C assuming a segregated bacterial population that initially comprizes a viable cell fraction of 0.3 and a non-viable fraction of 0.7 (for equations see Appendix). The relative ratios were estimated on the basis of colony

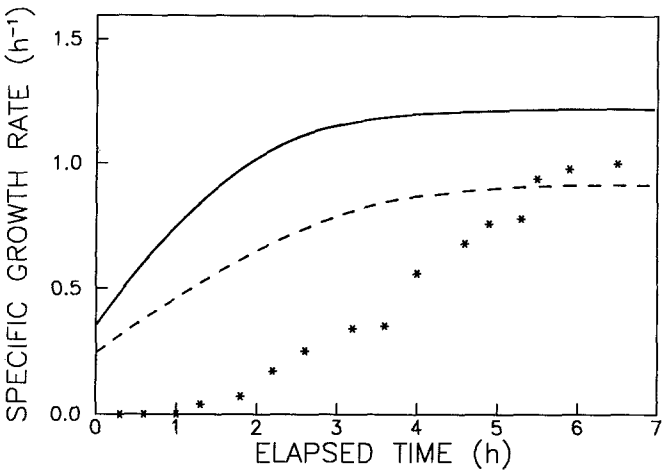

Fig. 3. Recovery of *K. pneumoniae* after 5 min heat shock at 48° C. Comparison between experimental data (*) and responses based on calculations assuming a segregated population consisting of an initially constant non-viable fraction of 0.7 and a viable portion of 0.3 which was able to grow at a constant rate of either 0.9 h^{-1} (*dashed curve*) or 1.22 h^{-1} (*continuous curve*)

counts observed on glucose agar. Calculations were performed for two different growth rate constants of the viable cell fraction: 1.22 h^{-1}, the maximum value at 35° C and 0.9 h^{-1}, which corresponded to the initial growth rate constant of the control. Neither of the simulations resulted in either reasonable quantitative or qualitative approximations of the experimental response.

Discussion and conclusions

The exposure of exponentially growing cultures of *K. pneumoniae* to various heat shocks results in both transitory reductions in the specific growth rate constant and significant lag phases, depending on shock intensity. Possible bases for such responses are that at superoptimal temperatures, bacterial growth is generally reduced (Esener et al. 1981) and, that step changes in growth temperature can result in pronounced time delays with respect to adjustment of the specific growth rate constant (Ryu and Mateles 1968; Ingraham 1987).

Since the maximum growth temperature for *K. pneumoniae* in minimal medium with glucose as sole carbon energy substrate is between 45° C and 46° C, response patterns to the heat shocks were divided into two groups (Franks et al. 1980), i.e., those in the superoptimal temperature range where balanced growth is still possible and those in the supermaximal temperature range where the maximum growth temperature is exceeded and growth does not occur. Short term heat shocks in the superoptimal temperature range result in recovery patterns which suggest that bacterial injury was insignificant, thereby confirming earlier results for *K. pneumoniae* when cells from a chemostat were heat shocked by passage through a heat exchanger (Heitzer et al. 1989). Although short term heat shocks in the superoptimal temperature region do not seem to cause major damage such temperatures can significantly alter the levels of many enzymes (Herendeen et al. 1979). These latter changes can occur very rapidly. Yamamori and Yura (1980) reported that the induction of synthesis of heat shock proteins in *E. coli* after a step increase from 30° C to 42° C was initiated within 60 s. Such energy consuming metabolic alterations probably take place at the expense of the maximum growth rate, an explanation consistent with the response observed here after subjecting *K. pneumoniae* to a heat shock at 45° C.

Heat shocks at supermaximal temperatures resulted in much more pronounced effects that were strongly temperature stress and exposure time dependent. Colony counts on minimal glucose agar for heat shocks at 48° C showed significant reduction in survival, suggesting that a part of the culture lost its capacity to recover, leading to the conclusion that recovery patterns must be interpreted by segregation of the bacterial population into a viable fraction and a non viable fraction (Hamer and Heitzer 1990). Calculations conducted for a 5 min heat shock at 48° C on this basis resulted in the prediction of much faster recovery than was, in fact, observed (Fig. 3), suggesting that the viable cell fraction must also have temporally lost its capacity to grow at its maximum rate, probably due to reversible damage. Earlier, Takano and Tsuchido (1982) concluded that growth delays in exponentially growing cultures of *E. coli*, after heat stress at supermaximal temperatures, were partially due to an increase in reversible injury to the viable fraction. For recovery after a supermaximal temperature shock, Daniels et al. (1984) reported that it took > 2 h for complete restoration of normal protein synthesis in *Halobacterium volcanii*. In the present experiments, the apparently non-viable fraction of the bacterial population present after heat shocks at 48° C should not be considered as a dead fraction, since recovery was found to be complete on protein rich plate count agar, on which the maximum growth temperature for *K. pneumoniae* is between 47° C and 48° C. In contrast, heat shocks at 51° C resulted in reduced viable counts on both agar media, indicating that this heat treatment was lethal to a fraction of the population. The previous history of bacteria before subjecting them to a heat shock can significantly affect recovery. Jenkins et al. (1988) cited prior starvation as enhancing thermal resistance relative to exponentially growing bacteria, whilst prior treatment at a superoptimal temperature when subjecting bacteria to supermaximal heat shocks enhances survival (Van Bogelen et al. 1989; Ramsey 1988). Another important question advanced by van Uden and Madeira-Lopes (1976), concerns the contribution of a non-viable fraction in a microbial population to substrate consumption. From the present study it is clear that the choice of an appropriate host organism, when a recombinant strain with a temperature regulated expression system is constructed, depends on both its optimum and its maximum temperature for growth, particularly when growth after heat shocking is required.

Appendix

The change in total biomass, x_t, during recovery after a heat shock for a segregated bacterial population comprizing a viable fraction x_v and a non-viable fraction x_n at time t is given by

$$x_t(t) = x_n + x_v(t). \quad \text{(A1)}$$

The viable cells contribution to growth a time t is given by

$$x_v(t) = x_v(t=0)\, e^{\mu t} \quad \text{(A2)}$$

where μ is the specific growth rate constant of the viable fraction. Combination of equation (A2) and (A1) results in:

$$x_t(t) = x_n + x_v(t = 0)\, e^{\mu t} . \quad \text{(A3)}$$

For a small time interval ($t_{n+1} + t_n$), where t_n and t_{n+1} are initial and final times respectively, the apparent growth rate μ_a at time $t_a = 0.5\,(t_{n+1} + t_n)$, is given by:

$$\mu_a(t_a) = [x_t(t_{n+1}) - x_t(t_n)]/[0.5(x_t(t_{n+1}) + x_t(t_n))(t_{n+1} - t_n)] \quad \text{(A4)}$$

Application of equation (A4) for different times allows predictions of the type shown in Fig. 3 to be made.

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