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The *Bacillus cereus* GerN and GerT protein homologs have distinct roles, in spore germination and outgrowth respectively.

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Running title: *Bacillus cereus* GerT protein and pH or Na^+ stress during outgrowth

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Summary

The GerT protein of *Bacillus cereus* shares 74% amino acid identity with its homolog GerN. The latter is a Na^+/H^+-K^+ antiporter that is required for normal spore germination in inosine. The germination properties of single and double mutants of *B. cereus* ATCC10876 reveal that unlike GerN, which is required for all germination responses that involve the GerI germinant receptor, the GerT protein does not have a significant role in germination, although it is required for the residual GerI-mediated inosine germination response of a *gerN* mutant. In contrast, GerT has a significant role in outgrowth; *gerT* mutant spores do not outgrow efficiently under alkaline conditions, and outgrow more slowly than wild type in the presence of high NaCl concentrations. The GerT protein in *B. cereus* therefore contributes to the success of spore outgrowth from the germinated state during alkaline or Na^+ stress.

Introduction

Bacillus cereus ATCC10876 spores germinate in inosine or in L-alanine as sole germinants (5). They require both GerI and GerQ germinant receptors for germination in inosine as sole germinant, whereas the GerL receptor is responsible for most of the response to L-alanine as sole germinant, with a smaller contribution from the GerI receptor (3). The GerN protein is needed for successful inosine germination, as spores of a *gerN* mutant of *B. cereus* ATCC10876 demonstrate a slow and abnormally-ordered germination response in inosine as sole germinant (24). The residual germination was very asynchronous, and some spores appeared to become phase dark before losing heat resistance (24). This GerN protein is a homolog of a widely distributed family of cation transporters ((9), and has been demonstrated to mediate Na^+/H^+ and Na^+/ H^+ -K^+ antiport activity when expressed at a low level in *E.*

coli (21). Such GerN-mediated antiport activity is therefore apparently required for the germination response mediated by the GerI germinant receptor in *B. cereus*. The role of such an ion transport protein in germination remains unclear, especially as other germinant receptors in the same strain do not require a functional GerN protein – for example, the *gerL*-dependent alanine response is almost identical to wild type in a *gerN* mutant (24). Both GerN and a closely related homolog are encoded in *B. cereus* ATCC 14579 (13), and in other members of the family, including *B. anthracis* (17) and *B. thuringiensis* (16). Both genes appear to be monocistronic and their coding sequences are widely separated on the genome. We have called this second *gerN*-like gene *gerT* (19). An entirely different (spore coat) protein in *B. subtilis* has been recently called *gerT* (7) , but that gene does not have a homolog in *B. cereus*, and as *B subtilis* does not encode an equivalent homolog of the *B. cereus gerN* or *gerT* genes, the nomenclature will not overlap. We describe here the spore germination and outgrowth phenotypes of mutants of *B. cereus* ATCC 10876 carrying an insertionally inactivated *gerT* gene. Resulting phenotypes suggest that the GerN and GerT proteins have distinct roles in germination and outgrowth.

Materials and Methods

Bacterial growth, media, strains.

Escherichia coli cloning strain TG1 and *B. cereus* ATCC 10876-derived strains were routinely cultured in L broth (Difco Bacto Tryptone 10 g l^{-1}, Difco Yeast Extract 10 g l^{-1}, NaCl 5 g l^{-1}, pH 7.2) or on L agar containing the appropriate antibiotics (for *E. coli,* ampicillin at 50 μg $ml^{-1}$and chloramphenicol at 30 μg ml^{-1}; *for B.cereus,* erythromycin and lincomycin at 1 and 25 μg ml^{-1} respectively, and kanamycin at 50 μg ml^{-1}). Spores of *B. cereus* were prepared in CCY medium and harvested and

washed at least 10 times in water as described previously (5). Oxoid Nutrient Broth (NB; pH 7.4) was used for outgrowth experiments.

B. cereus strains are listed in Table 1. *E. coli* K12 strains used for attempts at complementation of ion transport defects were the Na^+/H^+ antiporter-deficient strain Knabc (*chaA nhaA nhaB*) (15), and the potassium uptake-deficient strain TK2420 (Δ*kdpABC trkD1 ΔtrkA)* (11)*,* as already used in the study of *gerN* (21). Strain KNabc is defective in sodium efflux, and consequently is unable to grow at high concentrations of Na^+ (>75 mM) but will grow in a modified low sodium medium LBK (11) , which contains 10 mM NaCl and 50 mM KCl. To test Na^+ sensitivity, colonies of KNabc/pAS1 and KNabc/pGEM3zf+ were grown for 24h at 37 ^oC in 5 ml of LBK plus chloramphenicol, and the culture was then diluted 250 fold into LBK in which the NaCl concentration was varied, and grown for 16 hours at 37 ^oC.

Cloning and sequencing of *gerT* from *B. cereus* ATCC 10876. PCR primers based on the *B. anthracis* genome sequence data around BA0819 (*gerT*) were used to amplify the regions flanking the *gerT* gene of *B. cereus*, using High Fidelity *Taq* Extend (Boehringer Mannheim). The resulting *B. cereus*-derived PCR products were sequenced, and primers were designed for cloning *B. cereus gerT* without its promoter region, as a 1.1kb fragment. These were APS11 (*CTGGTCGAC*TAAAGGAGGAGCAGATGCTAT) and APS12 (*TTCGAGCTC*CTATCTATACAAAATATTTC), incorporating (italicised and underlined) *Sal*I and *Sac*I sites at their ends, respectively. Following restriction enzyme digestion, the PCR product was ligated into *Sal*I and *Sac*I digested pGEM3zf+, with *gerT* in the reverse orientation relative to the vector's *lac* promoter, but downstream of the T7 promoter. *E. coli* TG1 (a strain lacking T7 polymerase)

was transformed, yielding plasmid pAS1 for sequencing and complementation studies.

The fully overlapped sequence of the *gerT* region has been submitted to GenBank (Accession number EU789572). The monocistronic *gerT* locus would encode a 375aa GerT protein, with 99% amino acid identity to the equivalent protein in *B. anthracis*. A potential ribosome binding site is appropriately located upstream of the putative *gerT* start codon, and the *gerT* stop codon is followed by a potential rho – independent terminator.

Construction of *gerT* null mutants in *B. cereus*

Plasmid vector pSMUT lacks an origin of replication for *B. cereus* but carries a ColE1 replicon and a β-lactamase gene for amplification and selection respectively in *E. coli.* It is a derivative of pMUTIN4 (25), in which the *lac*Z gene has been removed and the ery/lin resistance gene replaced by a kanamycin resistance cassette. Primers APS7 (*CGTGAATTC*GGTAAGTTAATTGTTGGTTA) and APS8 (*AACGGATCC*CAATAACAATCGGCTGTGAA) were used to PCR amplify a 1.0kb internal fragment of *gerT*, from 108 base pairs downstream of the start of the *gerT* ORF to 76 base pairs before the stop codon of *gerT*. This PCR fragment was digested with *EcoR*I and *BamH*I , and ligated with *EcoR*I and *BamH*I digested pSMUT, yielding plasmid pAS3. *B. cereus* was electroporated with 2.5µg of pAS3 DNA, and transformants selected on NA containing kanamycin. Integration into the chromosome by a single crossover within the region of homology would interrupt the *gerT* gene, so that it encodes a protein truncated by 25 amino acids from the C terminus. The disruption of the *gerT* gene and expected novel junction fragments were confirmed by PCR in a transformant, named AM1631 (*gerT1::pSMUT).*

Construction of a *gerN, gerT* double mutant.

The *gerN* mutant used in this study was strain AM1421 (*gerN17::pMUTIN4*), in which the *gerN* gene was insertionally inactivated by integration of plasmid pMNAP, derived from pMUTIN4 by cloning of an internal fragment of the *gerN* gene, spanning bases 365-813 of the ORF; integration of this plasmid results in C-terminal truncation of GerN by 120 amino acids. The inactivated gene was checked by Southern blotting (23). This *gerN17::pMUTIN4* mutation was introduced into strain AM1631 (*gerT1::pSMUT*) by generalised transduction with CP51ts, as described previously (5). Transductants were selected for resistance to erythromycin and lincomycin, and screened for retention of the kanamycin resistance marker. Colony PCR confirmed the presence of the mutations in both genes. One transductant was retained and named AM1632 (*gerN17, gerT1*).

Germination experiments. Spore germination used washed spores, heat activated for 30 min at 70 ^{o}C in H_2O, and conditions were as described previously (3), unless otherwise stated. OD_{490nm} was measured at intervals on a Wallac Victor plate reader. For amino acid enhanced L-alanine germination, germination was initiated by a combination of a sub-germinal concentration of L-alanine (20 μM) plus histidine, proline or tryptophan at 10 mM, or tyrosine at 1 mM. Buffer conditions were those optimal for L-alanine as sole germinant - Tris HCl (10 mM), pH 8.9 with NH_4Cl (50 mM), and germination was at 30 ^{o}C. Spores were preincubated in the alanine racemase inhibitor O-carbamyl-D-serine (5 μg/ml) in germination buffer for 5 min before addition of germinants, to prevent any conversion of L-alanine to its competitive inhibitor D-alanine. For amino-acid enhanced inosine germination, germination buffer was TrisHCl (10mM) pH 8.0, NaCl (10mM) and spores were

germinated at 37 ^{o}C; inosine was used in this experiment at a just-subgerminal concentration (40μM), and the same amino acid adjuncts were added as above.

Results:

GerT complements the Na^+ sensitivity of an *E. coli* mutant.

Cloning the *gerN* gene into *E. coli* strains with deficient Na^+ /H^+ antiporter activity and K^+ uptake (KNabc and TK2420, respectively) was reported to complement their respective compromised phenotypes (21). A similar test was carried out for the homologous *gerT* gene (Fig. 1). Introduction of the vector plasmid pGEM3zf+ did not improve growth of the KNabc strain, which was unable to grow in concentrations of NaCl over 75 mM ; in contrast, KNabc/pAS1, containing a *gerT* gene cloned without an efficient promoter upstream, as had been done for *gerN* previously, was able to grow in NaCl concentrations up to 150 mM. These data suggest that GerT provides some additional capacity for Na^+ efflux. Attempts to introduce pAS1 into the K^+ transport-deficient *E. coli* strain TK2420 (10), were unsuccessful, so no predictions can be made about the potential for K^+ transport by the GerT protein.

Spore germination in single and double mutants in response to single germinants. Water-washed, heat activated spores of mutants carrying insertionally inactivated *gerN* and *gerT* genes, individually and in combination, were used in germination assays (Fig. 2). Germination was measured as the fall in OD of spore suspensions after exposure to inosine. Spores of both wild type and *gerT* mutant germinated rapidly at 0.1 mM and 1 mM inosine. As previously reported (24), the *gerN* mutant spores have a severe germination defect in inosine, but do still show

some residual response in high (1 mM) inosine; this residual response is dependent on GerT, as it is absent in a *gerN*, *gerT* double mutant. GerT is therefore not required for germination in inosine provided that GerN protein is available, but it can provide a partially functional substitute for GerN if the latter is missing from the spore. Germination in L-alanine under optimal conditions in the mutants was essentially identical to wild type (data not shown) – under these conditions, the receptor involved is GerL (3); therefore neither GerN nor GerT is required for germination involving the GerL receptor.

If the GerI receptor is required for a response to combinations of germinants, GerN is also required.

Although most members of the *B. cereus* family germinate in L-alanine and inosine, either individually or in combination, the range of germinant receptors encoded by different strains is variable, as consequently is the detailed germination behaviour. Unlike *B. cereus* ATCC14579 (12), which has a slightly different complement of germinant receptors, *B. cereus* ATCC10876 germinates in L-alanine plus aromatic amino acids, in a generally similar fashion to the so-called AEA , or aromatic-enhanced alanine, response of *B. anthracis* (8). In ATCC 10876, a sub-germinal concentration of L-alanine (20μM) is effective in combination with tryptophan or tyrosine, although there is no response with histidine or proline (Fig 3A). Spores of *gerI*, *gerL* or *gerN* mutants all fail to germinate in the alanine + tryptophan or tyrosine combinations, but *gerQ* and *gerT* mutants germinate like wild type ((19), data not shown). These data demonstrate a requirement for both GerL and GerI germinant receptors, consistent with previous observations for the equivalent response in *B anthracis* Sterne (8). The third receptor required in *B. anthracis*, GerS, is also

191 encoded in *B. cereus* ATCC10876 (2)) , but has not been mutated to test function.
192 Another germinant combination effective in *B. anthracis* is inosine plus aminoacids.
193 In *B. cereus* ATCC10876, sub-germinal (40 μM) inosine with either tryptophan or
194 histidine proves an effective germinant combination (Fig 3B). As is the case for
195 inosine as sole germinant, both GerI and GerQ receptors, and GerN, are required
196 here, but there is no requirement for the GerL alanine receptor, or for GerT (Fig 3C).
197 These data altogether suggest that in every case when the GerI receptor is required, so
198 also is GerN; the GerT protein does not compensate functionally for GerN in these
199 circumstances.
200
201 **GerT has a role in outgrowth in high salt and at alkaline pH.**
202 As *gerN* and *gerT* mutants germinate normally in L-alanine, it is possible to test for a
203 role of these genes in spore outgrowth. Spores were germinated in L-alanine in Tris
204 HCl buffer, harvested by centrifugation and resuspended in NB to allow outgrowth.
205 Spores of the parent strain, *gerN,* and *gerT* mutants all outgrew at similar rates in NB
206 (Fig 4B). In NB adjusted to pH 9.5 with NaOH, the wild type and *gerN* mutant
207 spores outgrew at the same rate, after a lag of ca 30 and 60 min respectively, but a
208 clear defect was observed for the *gerT* mutant spores, which did not outgrow
209 significantly within the period of the experiment. The *gerT* mutation also reduced the
210 ability of spores to outgrow in NB at pH 7.4 in the presence of additional 0.7 M NaCl
211 (Fig 4C); there was significant cell lysis, and no outgrowth above the initial OD
212 within 2.5 h. Unlike the situation in outgrowth, vegetative cells of these mutants all
213 grew at the same rate as wild type in NB at pH 9.5 (Fig 4D). Sensitivity to alkaline
214 pH in the *gerT* mutant is therefore limited to the outgrowing state. No difference was
215 seen in the behaviour of mutant and wild type during vegetative growth following salt

stress (data not shown). These data suggest that during vegetative growth, but not during outgrowth, alternative sodium efflux systems are likely to be present at a sufficient level to deal with these environmental stresses, although the nature of such Na^+ transport systems in the *B. cereus* family has not yet been explored.

Discussion:

Based on the phenotypes of *B. cereus* mutants, the related proteins GerN and GerT, both of which are likely to have Na^+/H^+ antiport activity, have primary roles in germination and outgrowth respectively, and as neither protein is required for NaCl resistance in vegetative cells, their role appears to be mainly in spore biology. There is some evidence that both *gerN* and *gerT* genes are expressed during sporulation; the *gerN* gene of *B cereus* is specifically sporulation expressed (20), from t_4 in sporulation, at a very low level; from *lacZ* fusion data its expression is approx one-third the level seen for the *gerI* operon, and one-hundredth that of *exsA,* which encodes a spore coat morphogenetic protein(1). A *lacZ* fusion to the *gerT* gene of *B. cereus* is not available, but the *gerT* gene of *B. anthracis* (BA 0819) has been reported from microarray data as late-sporulation expressed (14).

Role of GerT.

Both GerN and GerT are members of the CPA-2 monovalent cation:proton antiporter family of membrane transport proteins (18), and are homologs of the NapA family of proteins. The GerN protein behaves as a Na^+/H^+-K^+ antiporter, and is essential for normal GerI receptor function, possibly in the restoration of local ion balance (21). As yet, the transport capabilities of GerT have not been directly measured, but the NaCl and alkali sensitivity of the *B. cereus gerT* mutant during spore outgrowth, and

241 the ability of the cloned *gerT* gene to improve growth of a Na^+ transport-defective *E.*
242 *coli* mutant in complementation studies, both suggest a role in Na^+ efflux. As
243 described above, there is evidence for *gerT* expression during late sporulation, and
244 GerT must be present in dormant spores, as it is responsible for the phenotypic
245 difference between *gerN* spores and *gerN, gerT* double mutant spores in germination,
246 at a stage before de novo protein synthesis. Whether pre-existing GerT protein in the
247 germinated spore is sufficient for outgrowth, or whether in normal cells *gerT* is
248 expressed again during outgrowth, is not known.
249 The GerN and T proteins are significantly different in sequence, and separately
250 encoded in the genome. Consequently, the different roles of GerN and GerT proteins
251 in germination could reflect differences in their ion transport activity or specificity,
252 association with other proteins, or relative levels of protein. There are other reports
253 that precise functions can vary between homologs in the NapA family of transport
254 proteins (27).
255 The report that led to our initial study of GerN in *B. cereus* was that a related protein,
256 GrmA, is required for germination in *B. megaterium* ATCC12872 (22). A recent
257 report demonstrates that, in contrast, GrmA is not required for germination in the
258 apparently equivalent *B. megaterium* strain QMB1551 (4). Caution needs to be
259 exercised when considering different strains of a species, as genomic differences in
260 the spectrum of encoded and functional germinant receptors may be reflected in
261 different germination properties – for example the germination behaviour of *B cereus*
262 strains ATCC14579 and ATCC10876 show significant differences (12). It is not
263 obvious why, even within a single species, one germinant receptor (GerI) might be
264 strictly dependent on an ion antiporter like GerN, while other receptors are not. The

265 analysis of GerT has not clarified this issue, but has highlighted a second role for
266 proteins of this family, this time in outgrowth.
267
268 **Potential significance of GerT and GerN in the biology of the *B. cereus* family.**
269 Considering it as a naive cell, encountering a new environment , the newly-
270 germinated spore will have to deal with environmental stress, for example by salt or
271 alkali, and it appears that the GerT protein in the *B cereus* family is one protein that
272 has been recruited to this role. For example, when spores germinate in the alkaline
273 midgut of insects, or germinate in alkaline or saline soils, the GerT protein may be an
274 important contributing factor to the resumption of growth.
275 In an attempt to screen for potential germination defects in *gerT* mutant spores, we
276 studied amino acid enhanced germination in sub-germinal alanine and inosine. This
277 did not reveal any GerT function, but did demonstrate that GerN is important in any
278 circumstances where the GerI receptor is implicated in germination. In *B. anthracis*,
279 the GerH germinant receptor has been implicated in virulence (26). Apart from
280 sequence differences in the long repeat region near the N-terminus of GerHA and
281 GerIA, the GerH proteins are almost identical in amino acid sequence to the GerI
282 proteins of *B. cereus* ATCC10876. Unlike *B. cereus*, *B. anthracis* does not germinate
283 in inosine as sole germinant – the absence of *gerQ* in the *B. anthracis* genome may
284 explain this failure, so one does not need to invoke significant difference in function
285 between the GerI and GerH receptors. We predict therefore that GerN is likely to be
286 required in *B. anthracis*, for GerH receptor function. This is supported by recent
287 evidence (6) that *gerH*, *gerN* and *gerT* mutants were all enriched in large-scale
288 screens designed to detect defects in sporulation, germination or outgrowth of *B*
289 *anthracis*.

290

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Figure legends:

Figure 1. The effect of different Na^+ concentrations on the growth of *E.coli* strain KNabc, on introduction of the *gerT* gene of *B. cereus* . The OD of cultures was measured after 16 h growth at 37 ^oC. Precise growth conditions are described in the Materials & Methods. Symbols represent: ○, KNabc/pGEM3zf+, a strain containing the plasmid vector only; ●, KNabc/pAS1, containing the *gerT* gene cloned in pGEM3zf+.

Figure 2. Effect of *gerN* and *gerT* mutations on the germination response to inosine as sole germinant, measured by fall in OD of spore suspensions. Spore suspensions were incubated in 10mM Tris-HCl pH 8.0, 10mM NaCl at 37 ^oC over a range of inosine concentrations. **A**: Strain *B. cereus* ATCC10876 (*ger*$^+$); **B**: AM1631 (*gerT1*::pSMUT); **C**: AM1421 *(gerN17*::pMUTIN4); **D**: AM1632 (*gerN17*::pMUTIN4, *gerT1*::pSMUT). Symbols represent different inosine concentrations as follows: ◊, no inosine; □, 100μM; ○, 1mM. The data for 10μM inosine superimpose on the " no inosine" line.

Figure 3. Germination in sub-germinative concentrations of alanine or inosine, in combination with amino acid adjuncts.

A. Germination of spores of *B. cereus* ATCC10876, the Ger$^+$ parental strain, in L-alanine (20μM) plus other amino acids. Symbols are : □, no addition; ○, +10mM histidine; △, +10mM proline; ▲, +1mM tyrosine; ●, +10mM tryptophan.

316 B. Germination of spores of *B. cereus* ATCC10876, the Ger$^+$ parental strain, in 40μM
317 inosine plus amino acids. Symbols are as in A; □, no addition; ○, +10mM histidine;
318 △, +10mM proline; ▲, +1mM tyrosine; ●, +10mM tryptophan.
319 C. Germination of mutants in inosine (40μM) plus tryptophan (10mM). Symbols are:
320 ○, *B. cereus* ATCC10876, the Ger$^+$ parental strain; ◊, *gerL*; ▲, *gerT*. These three
321 graphs superimpose, and demonstrate complete germination. The following graphs
322 showed little or no germination, and are largely superimposed: ●, *gerQ*; △, *gerI*; □,
323 *gerN*.
324
325 Figure 4. Effect of *gerT* and *gerN* mutations on spore outgrowth and on vegetative
326 growth. Spores were germinated in L-alanine, then resuspended in fresh medium for
327 outgrowth. (●,WT, □, *gerN*; ○, *gerT*). **A.** Outgrowth in NB adjusted to pH 9.5. **B.**
328 Outgrowth in NB **C.** Outgrowth in NB plus 0.7M NaCl; **D.** vegetative growth in NB,
329 pH 9.5.

330

331 Table 1. *Bacillus cereus* strains used

Strain	Relevant genotype/antibiotic resistance	Source or reference
ATCC10876 UM20.1	*trp-1*, Str^r	(3)
AM1314	Tn917-LTV1::*gerIA5* Ery^r *trp-1* Str^r	(3)
AM1311	Tn917-LTV1::*gerQA2*Ery^r *trp-1* Str^r	(3)
AM1316	Tn917-LTV1::*gerLA1* Ery^r *trp-1* Str^r	(3)
AM1421	*gerN17*::pMUTIN4, Ery^r *trp-1* Str^r	(23)
AM1631	*gerT*::pSMUT Kan^r *trp-1* Str^r	This work
AM1632	*gerN17*::pMUTIN4, *gerT*::pSMUT Kan^r Ery^r *trp-1* Str^r	This work

332

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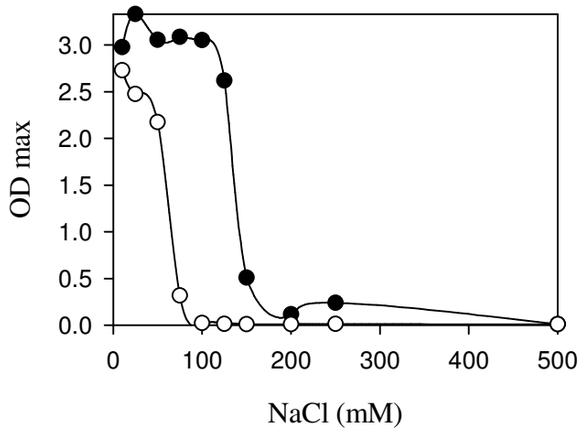

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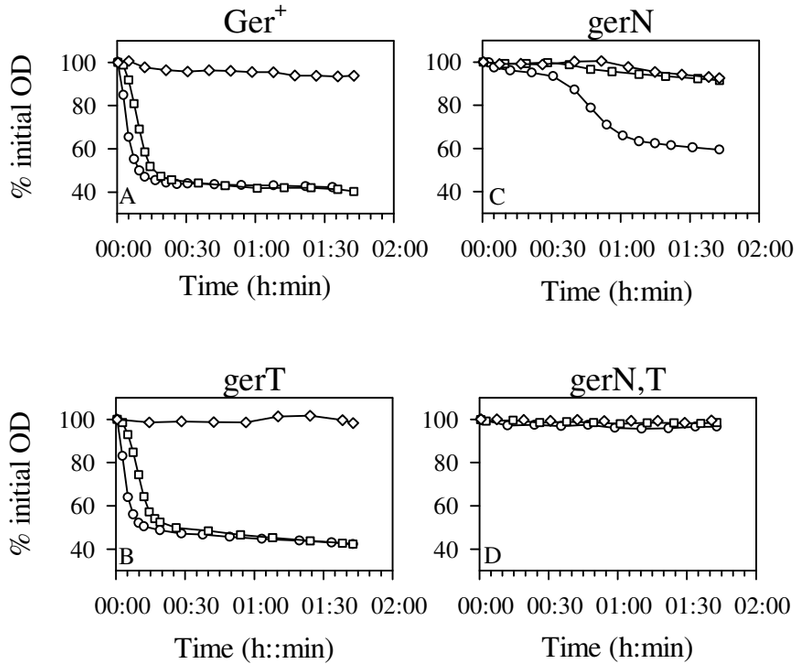
Figure 1, Senior&Moir



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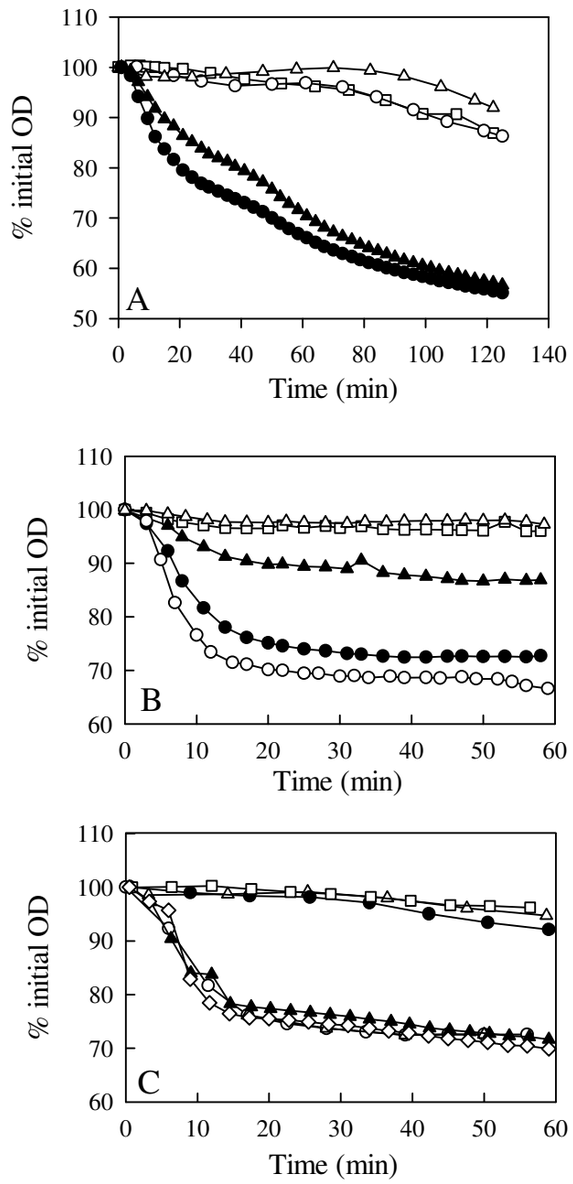
436 Figure 2 Senior&Moir



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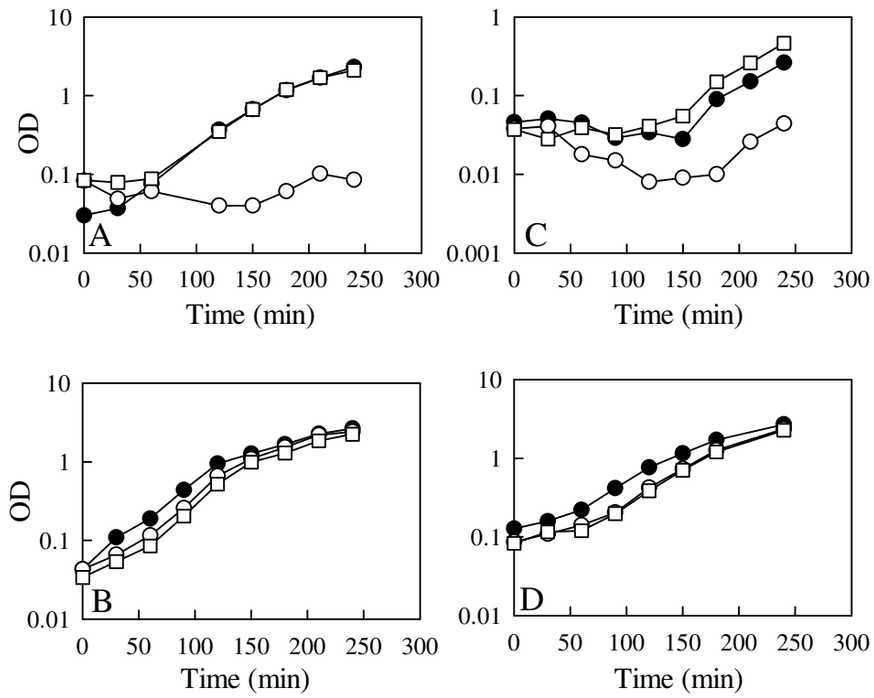

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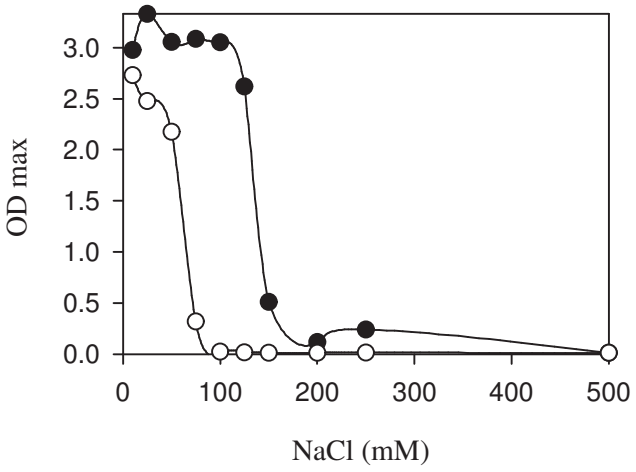
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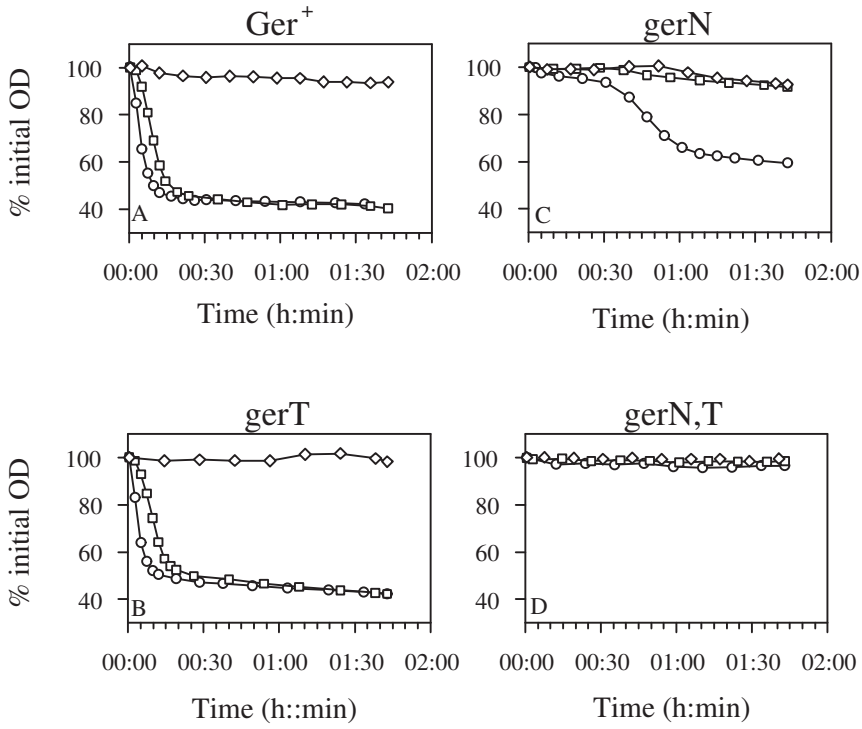
NaCl (mM)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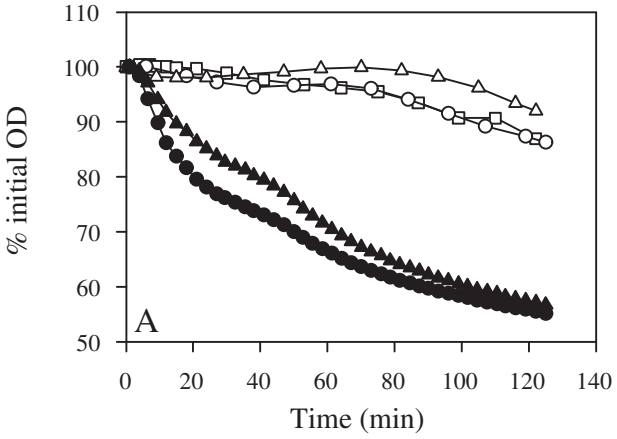
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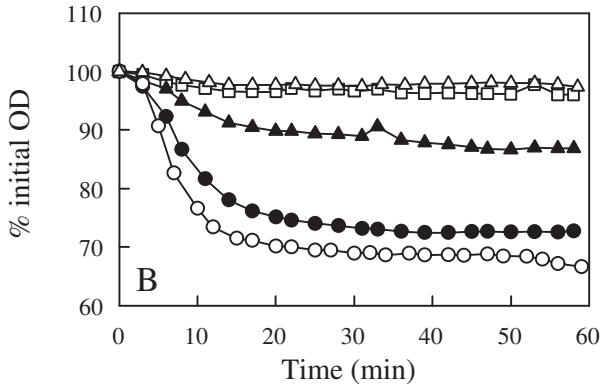
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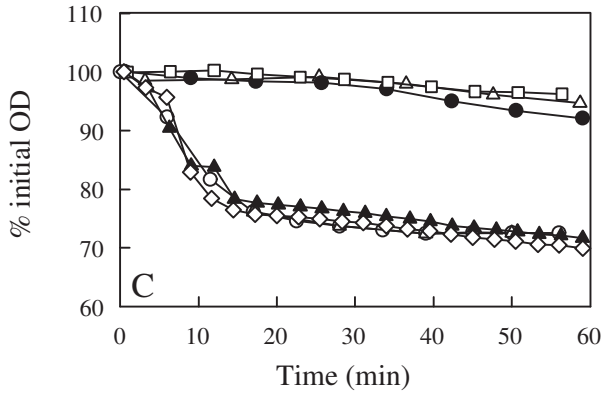
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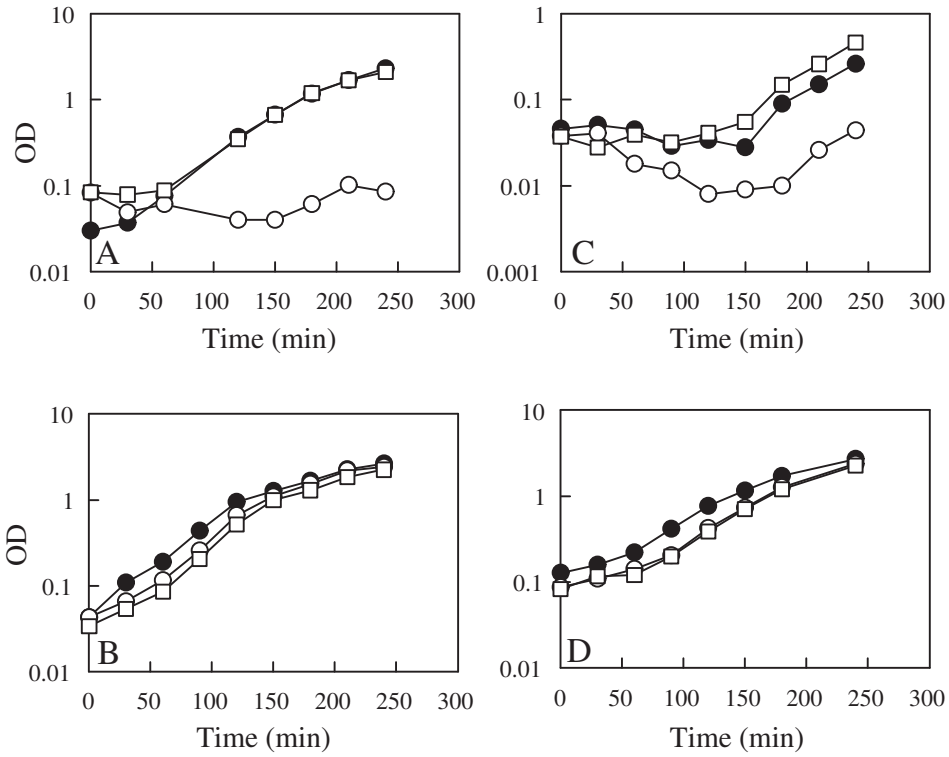
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