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2 **The genetically remote pathogenic strain NVH391-98 of the *Bacillus cereus***
3 **group represents the cluster of thermophilic strains**

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7 Sandrine Auger[§], Nathalie Galleron[§], Elena Bidnenko[§], S. Dusko Ehrlich[§], Alla Lapidus[#],
8 Alexei Sorokin^{§*}

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12 [§]Génétique Microbienne, INRA, Domaine de Vilvert, 78352 Jouy-en-Josas cedex, France

13 [#]DOE Joint Genome Institute, Walnut Creek, CA, USA

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16 **Key words:** *Bacillus cereus*, thermophilic, “*Bacillus cytotoxicus*”, temperate phages

17 **Running title:** Thermophilic growth in the *Bacillus cereus* group

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19 *** Corresponding author:**

20 **Alexei Sorokin**

21 **Génétique Microbienne, INRA Domaine de Vilvert, 78352 Jouy-en-Josas cedex, France,**

22 **tel: 33 1 34 65 27 24, fax: 33 1 34 65 25 21, email: alexei.sorokine@jouy.inra.fr**

1 **ABSTRACT**

2 Bacteria of the *Bacillus cereus* group are known to cause food poisoning. A rare
3 phylogenetically remote strain NVH391-98 was recently characterized to encode a
4 particularly efficient cytotoxin K presumably responsible for food poisoning. This pathogenic
5 strain and its close relatives can be phenotypically distinguished from other strains of the *B.*
6 *cereus* group by the inability to grow at temperatures below 17°C and by the ability to grow at
7 temperatures from 48 to 53°C. A temperate phage phBC391A2 residing in the genome of
8 NVH391-98 allows to distinguish the three known members of this thermophilic strain
9 cluster.

10

1 *Bacillus cereus* (*Bce*) group includes Gram-positive aerobic spore-forming bacteria
2 commonly found in soil and sometimes implicated in food poisoning. This opportunistic
3 pathogen causes gastrointestinal diseases manifested by diarrheic or emetic syndromes (5,
4 12). Practical importance of the *Bce* group studies is growing because of the increasing
5 number of related food poisoning cases, especially in developed countries. Since this problem
6 has an obvious relevance to the ability of some *Bce* strains to multiply in chilled products,
7 psychrotolerant strains have been in the focus (3, 4, 8, 22). It was shown that psychrotolerant
8 and mesophilic strains have optimal growth temperatures in the range of 25-35°C but
9 psychrotolerant strains can be distinguished by their ability to grow at 4-7°C, but not at 43°C
10 (14). Based on several distinctive features of psychrotolerant strains, including the presence of
11 a specific signature in the 16S rRNA sequences, a new species, *B. weihenstephanensis* (*Bwe*),
12 was proposed (14, 21). In contrast, the data available in regard to the ability of growth of the
13 *Bce* group bacteria at moderately high temperatures, that is close or slightly higher than 50°C,
14 are scarce, non-systematic and not sufficiently detailed. Some isolates were reported to grow
15 on plates at 55°C after 5 days of incubation (18). A strain NVH200 was able to grow up to
16 50°C in a liquid medium after a long lag phase of 72 h (1). Here we show that only very few
17 strains of the *Bce* group are able to grow at temperatures higher than 48°C. In fact this ability
18 seems to be restricted to a few strains represented by the genetically remote strain NVH391-
19 98, isolated from a severe food poisoning outbreak, which caused three fatal cases (15). This
20 strain is able to synthesize in elevated amounts a particularly efficient diarrheic cytotoxin K
21 (2, 7). NVH391-98 and its close relatives are the unique thermophilic isolates of the *Bce*
22 group, presumably representing another novel species.

23

24 **NVH391-98 represents a cluster of thermophilic strains.**

25 Psychrotolerant strains *Bwe* KBAB4 (20, 24) and *Bwe* WSBC10206 (14), obtained
26 from Dr. V. Sanchis (INRA, La Minière, France) and Prof. S. Scherer (IM, Freising,

1 Germany), were isolated from soil in France and Germany, respectively. Mesophilic strains
2 *Bce* ATCC14579 (9), obtained under the designation 6A5 from Dr. D.R. Zeigler (BGSC,
3 Columbus, USA), and *Bce* ATCC10987 (19), obtained from Dr. D. Lereclus (INRA, La
4 Minière, France), were of air and dairy origin from UK and Canada, respectively. The strain
5 NVH391-98, obtained from Dr. D. Lereclus, was isolated from a vegetable purée in France in
6 1998 (15). The strains INRA AF2, obtained under the designation INRA398, and NVH883/00
7 (10) were from Dr. M.-H. Guinebretiere (INRA, Avignon, France). *Bacillus subtilis* 168 was
8 from the laboratory collection. Standard manipulations with bacteria, phages and DNA were
9 done as described (16).

10 In a preliminary experiment we noted that NVH391-98 strain was able to grow rapidly
11 at 48°C, while none of other tested strains of the *Bce* group grew at this temperature. This is
12 illustrated by a simple plate growth test (Fig. 1). The strain *B. subtilis* 168, known to be able
13 to grow up to 52°C (11), was used as the high temperature growth control. This experiment
14 clearly indicated that at least at the high temperature there is a large difference in the growth
15 abilities of NVH391-98 strain compared to other representatives of the *Bce* group. To
16 characterize these differences quantitatively we examined the growth of five strains of the *Bce*
17 group, including two psychrotolerant strains, two mesophiles and NVH391-98. We used
18 three different liquid media, LB (Luria-Bertani), BHI (brain heart infusion) and YYT (LB
19 medium supplemented with 6 g/l bacto-tryptone and 5 g/l yeast extract), at temperatures
20 ranging from 8 to 55°C.

21 For each strain, an aliquot of the overnight culture was diluted 100 fold into fresh
22 medium. Growth was monitored by measuring the increase of the optical density at 600 nm
23 with an automatic cell growth analyzer (Bioscreen C, Labsystems). Microtiter plates
24 containing 300 µl per well were shaken continuously during 96 h.

1 Extrapolation of the growth profile curves for the psychrotolerant *Bwe* strains KBAB4
2 and WSBC10206 to the zero growth rates, using a model of Ratkowsky *et al* (17), indicated
3 the minimal theoretical growth temperature of 1 to 4°C. Experimentally, growth was detected
4 at the minimal temperature of 8°C (Fig. 2, the data for all tested strains and the equation
5 parameters are presented in Suppl. 1). Above the optimal growth temperature of 30-32°C, the
6 specific growth rates rapidly decreased with increase of temperature. Above 38°C no growth
7 was experimentally detected, while the theoretical maximal growth temperature was in the
8 range of 40-46°C. For the two mesophilic strains experimentally detected minimal growth
9 temperature was 12°C, while 2 to 7°C was the theoretical estimation, and an optimal growth
10 was observed between 35 and 40°C (Fig. 2, see Suppl. 1 for all data). The strain *Bce*
11 ATCC10987 was able to grow up to 47°C, while the *Bce* ATCC14579 strain did not grow
12 above 46°C. The theoretical maximal growth temperature for the mesophilic strains was in the
13 range of 49-53°C, again slightly higher (1°C) for the *Bce* ATCC10987. Multiple plate tests
14 for about 100 *Bce* group strains from our laboratory collection (not shown) confirmed the
15 general conclusion that almost all strains of the *Bce* group are able to maintain experimentally
16 detectable growth between 8 and 47°C and not beyond this range. If the Ratkowsky's model
17 adequately describes the whole range of temperature dependence of growth, these
18 temperatures can be extended to 2-53°C. Presumably, longer incubation times are needed to
19 detect the slow growth at the extreme temperatures. But also some growth induction
20 constraints can exist in our experimental conditions that do not allow to detect growth up to
21 the theoretical limits. These constraints can result in very long lag-times, hampering the
22 measurements of very slow exponential phase growth. That is why it is necessary to apply a
23 theoretical model for estimation of the extreme growth temperatures. The strain NVH391-98
24 did not grow at temperatures below 18°C but was able to grow up to 53°C (Fig. 2). The
25 optimal growth temperature was 40°C in LB, 42°C in YYT and 46°C in BHI, displaying a

1 relatively high dependence of growth ability of the strain on the media used. Theoretical
2 limits of growth, based on Ratkowsky's model, were 8-15°C and 58°C. The strain NVH391-
3 98 is therefore able to grow at temperatures 6-8 degrees higher than the mesophilic strains of
4 the *Bce* group.

5 **Phylogenetic remoteness of NVH391-98 and distinction from its close relatives**

6 The phylogenetic remoteness of the strain NVH391-98 to other representatives of the
7 *Bce* group was demonstrated recently (6, 13). Fig. 3A illustrates this using the Multiple Locus
8 Sequence Typing (MLST) schema proposed in Tourasse *et al* (23) for the extended set of
9 strains compared to that reported earlier (6). This comparison includes the sequences of the
10 strains NVH883/00 (GenBank acc. # # EF108377-383) and INRA AF2 (AF2), which are
11 closely related to NVH391-98 (6, 10) and have the similar thermophilic phenotype. We re-
12 sequenced the relevant loci for NVH883/00 and confirmed the differences with NVH391-98.
13 MLST and several other independently determined sequences (acc. ## EF108376, EF108384-
14 390) for the strain AF2 do not allow to distinguish it from NVH391-98.

15 We noted that after overnight propagation on solid media at 50°C and subsequent
16 longtime storage at room temperature the two strains, NVH391-98 and AF2 (but not
17 NVH883/00), show typical auto-lytic morphology, characterized by plaques-like clearings in
18 areas of dense growth on agar. Using double-layer agar assays, we found that the NVH391-98
19 strain produces bacteriophage (phage) that formed turbid plaques on the AF2, but not on the
20 NVH883/00 cell lawn. This phenotype allows to distinguish NVH391-98, AF2 and
21 NVH883/00 strains. We detected phage clear plaque forming mutants among turbid plaques
22 on AF2 with a frequency of approximately 2×10^{-4} (Fig. 3). Analysis of genomic sequence of
23 the strain NVH391-98 (acc. # NC009674) revealed two regions, near 2,690 and 3,010 kb,
24 designated phBC391A1 and phBC391A2, respectively, containing clusters of phage-related
25 genes and thus potentially encoding inducible prophages.

1 To identify the phage infecting the strain AF2, we isolated DNA from a clear plaque
2 forming phage mutant, designated phBC391B3vir (Fig. 3B), and analyzed it by *EcoRV*
3 digestion. Resulting profile corresponded the theoretical profile of the phage phBC391A2
4 DNA, rather than to that of phBC391A1 (not shown). Direct sequencing of this DNA by
5 primers specific to NVH391-98 chromosomal DNA produced readable chromatograms, with
6 the average signal strength 7-10 folds higher, only with primers corresponding to the
7 phBC391A2 (not shown). Moreover, we determined that the mutation causing the clear
8 plaque phenotype of phBC391B3vir was due to insertion of additional A into the stretch of
9 five A, resulting into a reading frame shift in the Bcer982969 gene encoding potential phage
10 repressor (Fig 3C). Therefore the three strains can be phenotypically distinguished by using
11 their different susceptibility to the phage phBC391A2. Only the strain NVH391-98 produces
12 this phage and only the strain AF2 can be used as an indicator strain to detect plaque forming
13 units.

14 In conclusion, the formal sequence based comparisons allow to consider the strain
15 NVH391-98 and its close relatives as a rather genetically remote species of the *B. cereus*
16 group. At present NVH391-98 and its two close relatives described here are the only known
17 strains of this group for which thermophilic growth is confirmed. We proposed earlier to
18 consider the strain NVH391-98 as a representative of a new species, for which the name
19 “*Bacillus cytotoxicus*“ was suggested (13). The existence of two closely related but different
20 strains INRA AF2 and NVH883/00 validates the novel species status of NVH391-98.

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1 **ACKNOWLEDGEMENTS**

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3 and V. Sanchis for the gifts of strains. The work was partially supported by the French
4 National Research Agency (project ANR-05-PNRA-013).

1 **FIGURE LEGENDS**

2 **Figure 1. Growth of different *Bacillus* strains at 30°C and 48°C.**

3 *B. subtilis* 168 is used as a positive control for high temperature growth. The strains *Bce*
4 ATCC14579, *Bwe* KBAB4 and NVH391-98 are of the *B. cereus* group.

5 **Figure 2. Growth of *Bacillus cereus* group strains at different temperatures.**

6 Growth rates (r) for representatives of psychrotolerant (*B. weihenstephanensis* KBAB4),
7 mesophilic (*B. cereus* ATCC14579) and thermophilic (“*B. cytotoxicus*” NVH391-98) strains
8 in different media are plotted against the growth temperature (T). The experimental points are
9 mean values of three independent experiments. Solid line shows non-linear least squares
10 approximation according to the Ratkowsky’s equation (17).

11 **Figure 3. Phylogenetic and phenotypic distinction of the “*Bacillus cytotoxicus*” strain**
12 **cluster.**

13 A. Neighbor-joining phylogenetic tree for a representative set of the *B. cereus* group strains.
14 Strain names and concatenated sequences are taken from the MLST database described in
15 (23), GenBank or genomic sequences. Vertical bars labeled C, T and W indicate the major
16 strain clusters according to (20). The cluster of the three strains closest to NVH391-98 is
17 labeled by Y. Sequences of four bacilli strains closest to the *B. cereus* clade, *B. subtilis* 168,
18 *B. amyloliquefaciens* FZB42, *B. licheniformis* ATCC14580 and *B. pumilis* SAFR-032, were
19 extracted from GenBank entries (acc. # # NC_000964, NC_009725, NC_006270 and
20 NC_009848) and used to represent an out-group. Completely sequenced strains are labeled by
21 seq. B. Turbid (left) and clear (right) plaques formed by the phage phBC391A2 and by its
22 mutant phBC391B3vir, respectively, on the INRA AF2 strain as indicator. C. Partial
23 nucleotide and corresponding amino-acid sequences of Bcer982969 gene in the phages
24 phBC391A2 (top) and phBC391B3vir (bottom). Location of this mutation in the putative

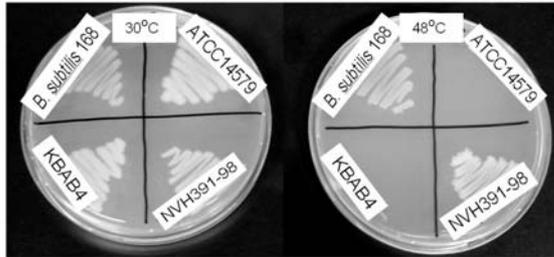
- 1 repressor gene of phBC391A2 proves the identity of phBC391B3vir as a clear plaque mutant
- 2 of the former phage.
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Figure 1



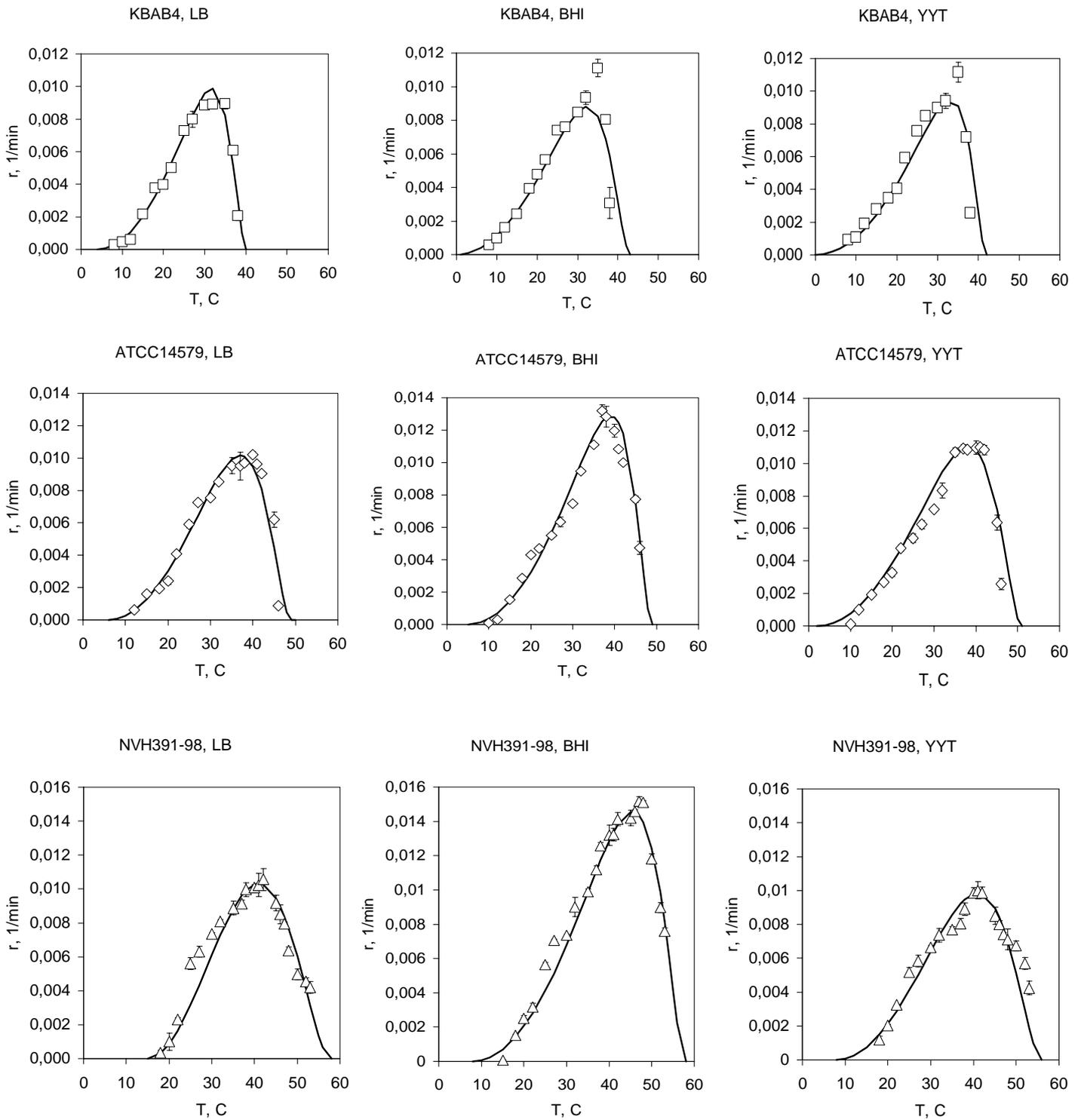
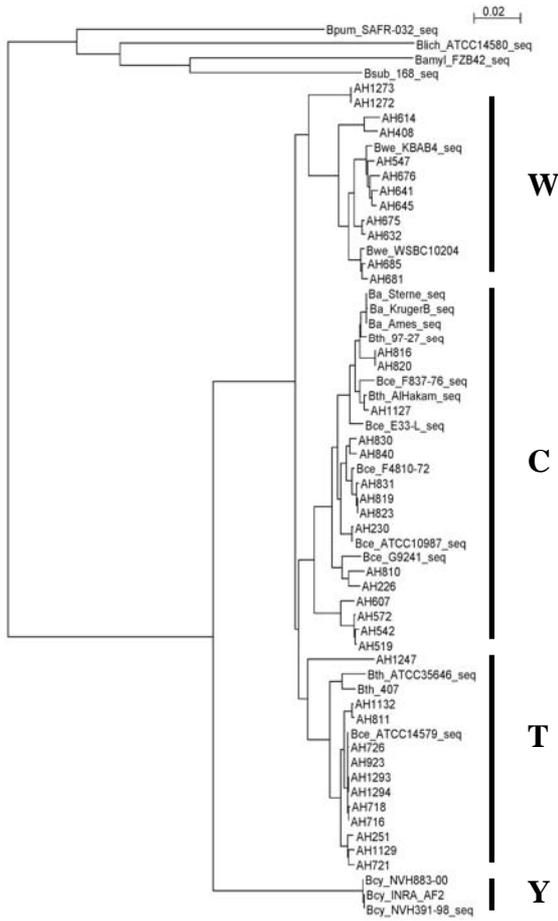


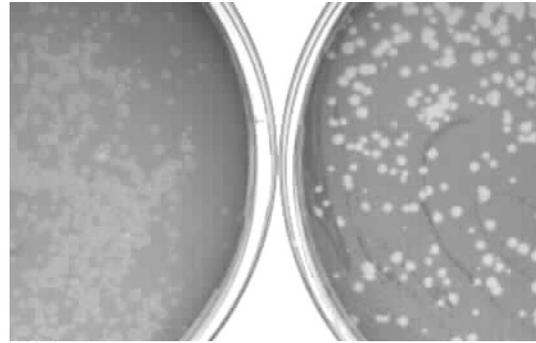
Figure 2

Figure 3

A



B



C

...AACCTTGGGTATGGGGAAAAAATAGTCTATATAGA...
... T L G M G K N S L Y R ...

...AACCTTGGGTATGGGGAAAAAATAGTCTATATAGA...
... T L G M G K K ter

Supplemental material.

Growth of *Bacillus cereus* group strains at different temperatures.

Growth rates for representatives of psychrotolerant (*B. weihenstephanensis* KBAB4 and WSBC 10206), mesophilic (*B. cereus* ATCC14579 and ATCC10987) and thermophilic (“*B. cytotoxicus*” NVH391-98) strains in different media (LB, BHI and YYT) were measured in the whole range of growth temperatures. Three independent growth experiments were done for each temperature, each medium and each strain. The non-linear least squares approximation was done according to the Ratkowsky’s equation (1). The equation :

$$\sqrt{r} = b(T-T_{min}) \{1 - \exp [c(T-T_{max})]\},$$

where r (min^{-1}) is the growth rate and T ($^{\circ}\text{C}$) is the growth temperature, contains four adaptable parameters : T_{min} , T_{max} , b and c .

T_{min} and T_{max} correspond to the minimal and maximal growth temperatures.

Since the equation is not linear, the initial estimation of least square regression is needed. For this the parameters b and T_{min} are first estimated from the low-temperature part of the curve.

The equation can be rearranged as :

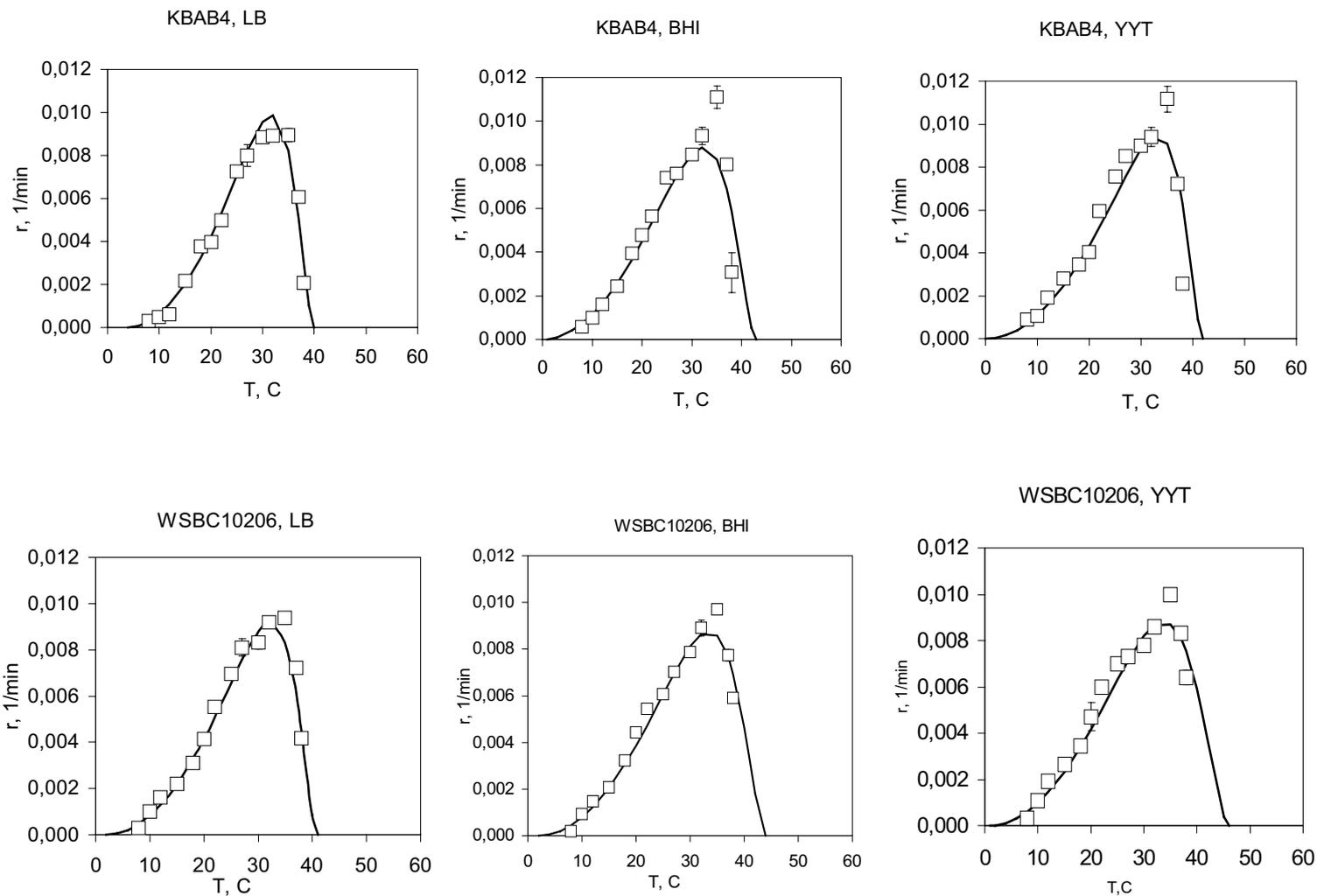
$$c(T-T_{max}) = \ln [1 - \sqrt{r} / b(T-T_{min})],$$

From this new equation c and T_{max} are estimated using the high temperature part of the curve. Random variation of the four estimated parameters allows then to find the optimal least square regression. The found parameters b , c , T_{min} and T_{max} for all five strains and three media are listed below. Figure shows the experimental points, which are the mean values of three independent experiments for each temperature, and non-linear least squares approximation according to the Ratkowsky’s equation (solid lines).

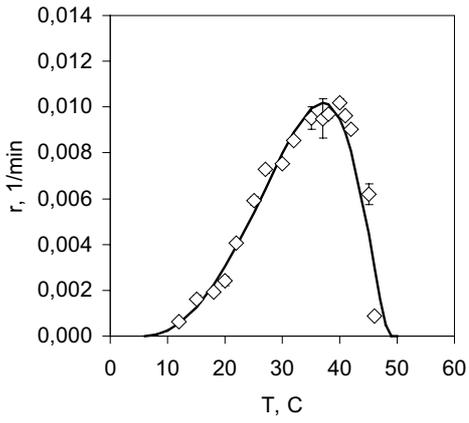
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Parameters of Ratkowsky's et al equation (1) for growth of different strains in liquid media.

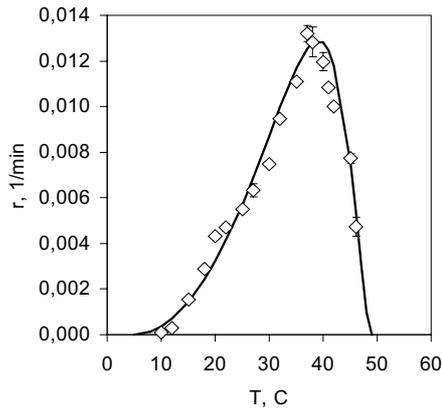
strain	medium	b	Tmin, °C	c	Tmax, °C
<i>B. weihenstephanensis</i> KBAB4	LB	0.0041	4	0.25	40
<i>B. weihenstephanensis</i> KBAB4	BHI	0.0034	0	0.18	43
<i>B. weihenstephanensis</i> KBAB4	YYT	0.0033	0	0.25	42
<i>B. weihenstephanensis</i> WSBC10206	LB	0.0036	2	0.2432	41
<i>B. weihenstephanensis</i> WSBC10206	BHI	0.0035	2	0.18	44
<i>B. weihenstephanensis</i> WSBC10206	YYT	0.0035	1	0.139	46
<i>B. cereus</i> ATCC14579	LB	0.004	6	0.14	49
<i>B. cereus</i> ATCC14579	BHI	0.0038	5	0.2115	49
<i>B. cereus</i> ATCC14579	YYT	0.0035	2	0.138	51
<i>B. cereus</i> ATCC10987	LB	0.0039	6	0.13	50
<i>B. cereus</i> ATCC10987	BHI	0.003	2	0.1572	53
<i>B. cereus</i> ATCC10987	YYT	0.0043	7	0.0922	52
" <i>B. cytotoxicus</i> " NVH391-98	LB	0.0072	15	0.046	58
" <i>B. cytotoxicus</i> " NVH391-98	BHI	0.0038	8	0.1501	58
" <i>B. cytotoxicus</i> " NVH391-98	YYT	0.004	8	0.0925	56



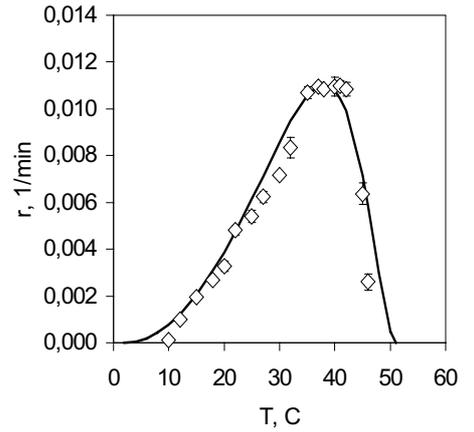
ATCC14579, LB



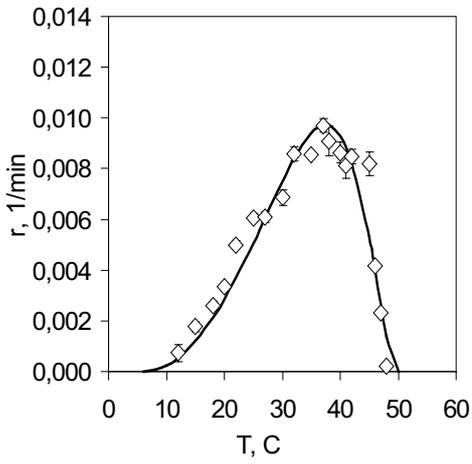
ATCC14579, BHI



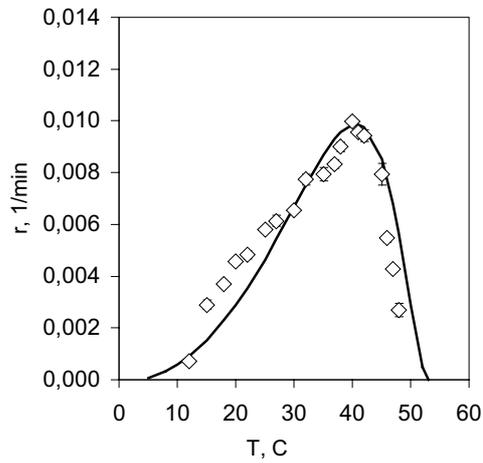
ATCC14579, YYT



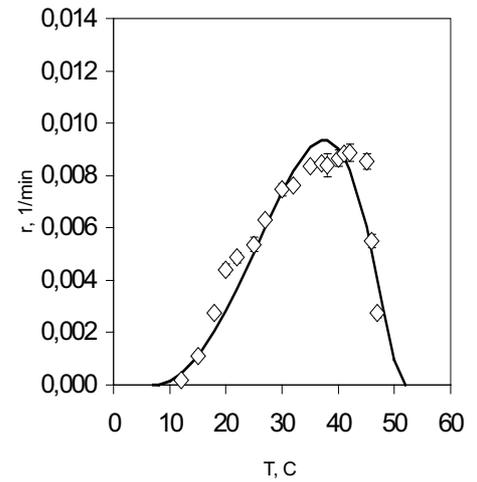
ATCC10987, LB



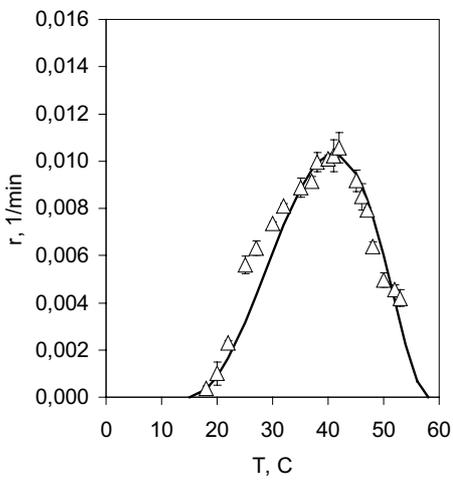
ATCC10987, BHI



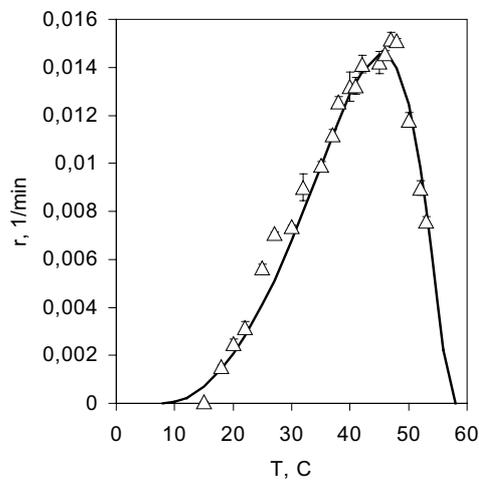
ATCC10987, YYT



NVH391-98, LB



NVH391-98, BHI



NVH391-98, YYT

