Isolation and Partial Characterization of a Temperature-sensitive Mutant of *Bacillus subtilis* Impaired in the Development of Competence for Genetic Transformation

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SUMMARY

A temperature-sensitive *Bacillus subtilis* mutant (Com^{ts}M8) has been isolated which is defective in genetic transformation at the restrictive temperature (42 °C). The mutant was selected on the basis of its transforming properties at 42 and 28 °C on agar plates spread with DNA. During growth at 37 °C the mutant shows normal development of transformability (competence), whereas at 42 °C competence is very low or completely absent. At 42 °C the mutant is not transfectable with DNA from bacteriophage ϕ 29, but susceptible to normal infection with the complete bacteriophage, indicating that the ability to take up exogenous DNA is impaired at this temperature.

In addition to the loss of competence the mutant shows a changed growth pattern at the restrictive temperature: cultures grow initially at the same rate as cultures of the parental strain up to the late exponential phase of growth and then abruptly change over to a much lower growth rate, simultaneously producing shorter cells than the parental strain. From a genetic analysis evidence has been obtained suggesting that the two altered properties of the mutant, loss of competence and a changed growth pattern at 42 °C, are due to the same mutation.

When the mutant is shifted during growth from the restrictive to the permissive temperature, its subsequent pattern of competence development is similar to that displayed by the parental strain after such a shift. Likewise, the response to an opposite shift is also similar in both strains. Only when the mutant is grown continuously at 42 °C, including the overnight incubation period, is the mutant genotype expressed completely.

The results suggest that at 42 °C the mutant is blocked in a relatively late step in the development of competence.

INTRODUCTION

Bacteria can be genetically transformed by exogenous DNA only after having developed competence (Hayes, 1968). Recently, it has been shown by Dooley, Hadden & Nester (1971) that, before acquiring competence, *Bacillus subtilis* cells pass through a precompetent phase starting 90 to 180 min before the appearance of competence. The precompetent state involves a changed pattern of cellular synthesis, which is characteristic of a low growth rate: a decreased rate of DNA synthesis and a decreasing ratio of ribonucleic acid to protein

synthesis. Indirect evidence supporting the existence of the precompetent state had been provided previously by Kretschmer (1967, 1968, 1969).

Several investigators have reported the isolation of non-conditional competence-negative mutants (Young & Spizizen, 1963; Young, Spizizen & Crawford, 1963; Caster, Postel & Goodgal, 1970; Prozorov, Kalinina & Schilina, 1971). We set out to isolate temperature-sensitive competence mutants, which are advantageous in that their deficiency can be provoked by changing the experimental conditions. Furthermore, such mutants are well suited for genetic analysis and have the special advantage that the effects of lethal mutations in genes controlling competence can be studied.

This paper describes the isolation procedure for such mutants and a partial characterization of one of them (mutant Com^{ts}M8) is presented.

METHODS

Strains

Bacteria. The parental *Bacillus subtilis* strain 3G-18 carrying three unlinked auxotrophic markers *trpC*₂, *purA* and *met* (tryptophan-, adenine- and methionine-requiring) is a derivative of strain 168 *trpC*₂ MARBURG (for the isolation of strain 3G-18, see Joenje, Gruber & Venema (1972)).

Bacteriophage. ø29 was kindly provided by Dr G. A. Wilson (Rochester, New York, U.S.A.).

Media

WB medium: Spizizen's minimal salts (Spizizen, 1958) plus 0.5% glucose, 50 µg/ml (each) of nine competence-enhancing amino acids (Wilson & Bott, 1968) which include tryptophan and methionine (all L-isomers), adenine (20 µg/ml) and 2.5 mM-MgSO₄. Minimal medium: Spizizen's minimal salts plus 0.5% glucose. Minimal agar: 1.75% agar (Difco) in Spizizen's minimal salts plus 1% glucose, the growth requirements (20 µg/ml) and amino acids for stimulation of growth (competence-enhancing amino acids 10 to 50 µg/ml each, or casein hydrolysate 200 µg/ml). TBAB agar: 3.3% Tryptose Blood Agar Base (Difco). Top agar for plating ø29 infectious centres: 1% Tryptone (Oxoid), 0.8% NaCl, 0.6% agar (Difco) and 0.6% glucose. All media were prepared with glass-distilled water. All chemicals used for the preparation of the media were purchased from BDH Ltd (Poole, Dorset), unless stated otherwise. Pancreatic deoxyribonuclease was from Miles Seravac (PTY) Ltd (Maidenhead, Berkshire).

DNA isolation

Bacterial DNA was isolated from tryptophan-independent cultures according to a modification of Kirby's procedure (Kirby, 1957) as described by Venema, Pritchard & Venema-Schröder (1965). ø29 DNA was isolated as described previously (Joenje *et al.* 1972). DNA concentrations were measured by the diphenylamine reaction as described by Burton (1956).

Assay of growth and competence development

Recipient cultures were grown to competence as follows. An overnight culture in WB medium was diluted into fresh prewarmed WB medium to 1×10^7 to 3×10^7 colony-formers/ml (corresponding to an E_{450} of approx. 0.2, see below) and subsequently grown under standard conditions. Where indicated, cultures were started from frozen (-85°C) log-phase

bacteria. Growth was followed by measuring the absorbance at 450 nm (E_{450}) in a Zeiss Spectrophotometer model PMQ II. At densities higher than 1.0, samples were diluted ten-fold before being measured. Development of competence was followed by transforming 0.9 ml samples with 3 to 5 $\mu\text{g/ml}$ *trpC*₂⁺ DNA (in 0.1 ml) usually during 30 min, unless stated otherwise. Uptake of DNA was terminated by the addition of 10 μl deoxyribonuclease (2 mg/ml) in 1 M-MgSO₄ and 5 min additional incubation. Transformants were scored on plates without tryptophan and supplemented with 0.02% casein hydrolysate. Dilutions were made in minimal medium. Transfectability was measured by exposing bacteria to 20 $\mu\text{g/ml}$ ϕ 29 DNA; transfectants were plated on TBAB plates with 2.5 ml top agar plus about 5×10^7 indicator cells.

*Selection of temperature-sensitive competence (*Com*^{ts}) mutants*

Exponentially growing cultures of 3G-18 in WB medium at 37 °C were treated for 90 min with 50 μg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemicals Co., Inc. Milwaukee, Wisconsin, U.S.A.)/ml. After washing with minimal medium the bacteria were grown in fresh WB medium during 4 to 5 h at 37 °C for mutant segregation. They were then plated on non-selective plates to give 100 to 200 colonies/plate and incubated at 42 °C. After the colonies had grown to a diameter of 1 to 1.5 mm each plate was replica-plated on to two plates lacking tryptophan but spread with DNA (approx. 30 $\mu\text{g/plate}$) extracted from *trpC*₂⁺. One series of replicas was then incubated at 28 °C, the other at 42 °C. After incubation overnight the majority of the colonies had grown on the DNA plates; those colonies growing on the 28 °C plates but lacking from the 42 °C plates were picked up from the master plate and assayed for their transformability in liquid medium at 37 and 42 °C. In this way 15 temperature-sensitive transformation-negative mutants were obtained; the majority of the mutants obtained by this method appeared to be of the non-conditional type, when retested in liquid medium. The mutant described in this paper was designated *Com*^{ts}M8.

RESULTS

*Transformability of 3G-18 relative to *Com*^{ts}M8 as a function of temperature*

To determine the temperature range in which the mutant changes its phenotype we compared the maximum transformation frequencies of *Com*^{ts}M8 cultures measured during one growth cycle with those of parental cultures at various temperatures between 34 and 42 °C, including the overnight period of growth. Fig. 1 shows that the temperature range in which the transition from wild-type to mutant phenotype occurs is very narrow. At temperatures in excess of 40 °C the mutant's transformability is affected drastically, whereas the parental strain is only moderately affected, resulting in a 270-fold difference. On the basis of these results we chose 37 and 42 °C as the permissive and restrictive temperatures, respectively.

Effect of temperature during incubation overnight on development of competence

To assess the effect of temperature during the overnight period of growth on the mutant and parental phenotype, overnight cultures of both 3G-18 and *Com*^{ts}M8 were grown at 37 °C and at 42 °C and after dilution into fresh medium cultured at 37 °C as well as at 42 °C. Fig. 2, in which only the peaks of competence have been presented, shows that the mutant's transformability at 37 °C following overnight growth at 42 °C is almost the same as that of the parental strain. This level of competence is equal to the level reached at 37 °C following overnight growth at 37 °C (not shown in Fig. 2). In the reverse case, growth overnight at

Fig. 1. Transformability of 3G-18 relative to Com^{ts}M8 as a function of temperature. Maximum transformation frequencies of 3G-18 and Com^{ts}M8 were determined during one growth cycle at various temperatures in the interval 34 to 42 °C. Overnight cultures were incubated at the corresponding temperatures. The ordinate values represent the ratio of the maximal transformation frequency of 3G-18 to that of Com^{ts}M8 determined at various temperatures.

37 °C followed by growth at 42 °C, the mutant transforms 10 times less well than strain 3G-18. The mutant's transformability is almost completely suppressed if subjected to the competence regimen at 42 °C following previous growth also at 42 °C. Additionally, in this experiment the mutant appears to develop its (poor) wave of competence at a later time than the parental strain. The figure also shows that the parental strain is less transformable at the elevated temperature than at 37 °C. However, transformability of Com^{ts}M8 cells is much more drastically affected.

Competence for transformation and ø29 transfection

To establish whether the Com^{ts}M8 mutant is blocked in the uptake of donor DNA or in its ability to integrate the DNA, we tested the mutant's capability of propagating phage progeny upon incubation with infectious DNA from bacteriophage ø29. This test seems to be unambiguous since ø29 transfection has been shown to be independent of the host's recombinational system (Spatz & Trautner, 1971). Fig. 3 shows that at the restrictive temperature the transfecting activity of ø29 DNA is also strongly reduced in the mutant when compared to that in strain 3G-18. Since Com^{ts}M8 produces normal amounts of phage progeny following infection with ø29 particles at the restrictive temperature (results not shown), we conclude that the mutant is not transformed at 42 °C because it is unable to take up the DNA. This experiment does not exclude the possibility that the mutant has an additional defect in its recombinational system; however, this seems unlikely since we have strong evidence (see below) that the transformation-deficiency is due to only one mutation.

Development of competence in relation to growth

Fig. 4 shows the growth and competence development of the parental and the mutant strains at 42 and 37 °C. Compared with 3G-18 the mutant shows a normal growth pattern at

Fig. 2. Effect of temperature during overnight incubation on competence development of 3G-18 and Com^{ts}M8. Overnight cultures of 3G-18 and Com^{ts}M8 were grown at 42 °C (A and C) or at 37 °C (B) in WB medium and diluted into fresh medium. Transformability was assayed during subsequent growth at 42 °C (B and C) or 37 °C (A) by transforming samples at hourly intervals (see Methods). Only peaks of transformability are shown. ○—○, 3G-18; △—△, Com^{ts}M8. In this and subsequent Figures the times indicated refer to the addition of donor DNA.

37 °C and also develops a normal (even higher) level of competence. At 42 °C, however, together with the loss of transformability, the mutant shows a changed growth pattern: at the end of the exponential phase of growth the growth rate decreases abruptly and becomes almost zero. Light microscopic examination of the mutant and parental bacteria at various times during growth shows a significant difference in bacterial length in samples taken after the interruption in the growth curve of the mutant has occurred. Com^{ts}M8 bacteria appear to be shorter than the parental bacteria, especially during the postexponential phase (see Fig. 5). In addition, the length of Com^{ts}M8 bacteria appears, from the standard deviations, to be less variable.

Number of mutations involved in the Com^{ts}M8 phenotype

For further analysis of the mutant it was essential to know whether the loss of competence and the changed growth pattern were due to the same or to different mutations. To examine this, we made use of the finding that mutant bacteria, as a result of their depressed growth

Fig. 3

Fig. 4

Fig. 3. Transformability and transfectability of 3G-18 and Com^{ts}M8 as a function of growth time at 42 °C. Overnight 42 °C cultures of both strains were diluted at time zero into fresh medium pre-warmed to 42 °C and grown at 42 °C. Transformability and transfectability were assayed as described in Methods. ----, Transfection frequency (% plaque-forming units); —, transformation frequency (% *trpC*₂⁺ transformants). ○, 3G-18; △, Com^{ts}M8.

Fig. 4. Growth and competence development of 3G-18 and Com^{ts}M8 at 37 °C and 42 °C. Frozen 42 °C-log phase cells of 3G-18 and Com^{ts}M8 were quickly thawed and diluted at time zero into fresh media at 37 °C (—) and 42 °C (----). Growth (a) and competence development (b) were determined as described in Methods. ○, 3G-18; △, Com^{ts}M8.

rate after the exponential phase of growth, form smaller colonies than the parental bacteria on agar plates incubated at 42 °C. Com^{ts}M8 bacteria were made competent at 37 °C and transformed during 30 min with a saturating concentration of DNA extracted from the prototrophic wild-type strain. After allowing segregation for 4 h the bacteria were spread on plates selective for *met*⁺ transformants and incubated at 42 °C. In the population of *met*⁺ colonies we determined the frequency of cotransformation for the *trpC*₂⁺ marker and the frequency of large colonies. These two frequencies appeared to be equal (Table 1). Apparently, the probability of incorporating the *trpC*₂⁺ marker is equal to the probability of incorporating the property determining wild-type growth, indicating that the changed growth pattern of the mutant is caused by one mutation. Because the frequency of large colonies within the population of *trpC*₂⁺ *met*⁺ colonies is roughly equal to the frequency in the total *met*⁺ population, it is concluded that the growth pattern-mutation is not linked to the *trpC*₂ marker. Ten large *met*⁺ colonies were tested in liquid medium and all appeared to have recovered their ability to develop competence at 42 °C. Ten small colonies tested similarly continued to show the mutant phenotype. This favours the conclusion that one mutation is involved causing both the changed growth pattern and the loss of transformability at

Fig. 5. Distribution of cell lengths of 3G-18 and *Com^{ts}M8* as a function of growth time at 42 °C. Overnight 42 °C cultures of 3G-18 and *Com^{ts}M8* were diluted into fresh medium and grown at 42 °C (inset: growth of 3G-18, x—x; and of *Com^{ts}M8*, o—o). The arrows indicate the times when samples were removed (1 to 5), concentrated by centrifugation and frozen at -85 °C. For phase-contrast microscopic examination the samples were quickly thawed and a few drops were spread on glass slides with a thin layer of minimal agar previously dried at 37 °C for 45 min to prevent motion of the bacteria. Lengths of 120 to 200 bacteria per sample were measured from photographs (the final magnification was 6000×). 3G-18: open, *Com^{ts}M8*: hatched histogram. The mean cell lengths ($\bar{l}$) and the standard deviations (s) for each sample are shown.

Table 1. *Number of mutations involved in Com^{ts}M8 phenotype*

Com^{ts}M8, made competent at 37 °C, was transformed with DNA from a prototrophic wild-type strain and spread on plates without methionine after 5 h of segregation at 37 °C. Within the population of *met*⁺ colonies the frequency of large colonies was determined as well as the frequency of colonies being transformed for the *trpC*₂⁺ marker (by replica-plating).

Experiment	Number of <i>met</i> ⁺ colonies screened	Frequency of large colonies (% of total)	Frequency of <i>trpC</i> ₂ ⁺ colonies (% of total)	Frequency of large <i>trpC</i> ₂ ⁺ colonies (% of total <i>trpC</i> ₂ ⁺ population)
1	2689	6.8	7.0	9
2	1675	4.7	4.7	not assayed

Fig. 6 (a), (b). Effect of temperature shifts on growth and competence development of Com^{ts}M8. Frozen 42 °C-log phase cells of Com^{ts}M8 were quickly thawed and diluted into fresh medium, whereafter growth (a) and competence development (b) were followed at 42 °C (culture 1, ○—○). Shortly after the exponential phase of growth had ended part of the culture was shifted to 37 °C (indicated by arrow A; culture 2). At two times, indicated by the arrows B and C, part of culture 2 was shifted back to 42 °C (cultures 3 and 4, respectively). Growth and competence development of all four cultures were followed (1, ○—○; 2, ●—●; 3, ×—×; 4, △—△). Samples were transformed for 15 min followed by 5 min of incubation with deoxyribonuclease before plating.

Fig. 6 (c). Effect of temperature shifts on competence development of 3G-18. Competence development of strain 3G-18 was followed during growth at 37 °C (culture 1, ●—●). At the times indicated by the arrows A and B a part of culture 1 was shifted to 42 °C (cultures 2, +—+, and 3, ○—○, respectively). Competence development was followed by transforming samples during 15 min followed by 5 min of incubation with deoxyribonuclease.

42 °C, although the possibility of two very closely linked mutations cannot be excluded entirely.

Effect of shifting the temperature on development of competence

In the second paragraph of the Results section we showed that overnight incubation of Com^{ts}M8 at the permissive temperature permits partial expression of the wild-type phenotype during the subsequent growth cycle at the non-permissive temperature. In the following experiments the effect of shifting the temperature during the postovernight incubation period on competence development in both the mutant and the parental strain was studied.

Fig. 7 (a). Response of Com^{ts}M8 to a temperature shift to 37 °C as a function of growth time at 42 °C. An overnight 42 °C culture of Com^{ts}M8 was diluted into fresh medium to an E_{450} of 0.3, after which competence development at 42 °C was followed (○—○). At hourly intervals, starting 3 h after dilution of the overnight culture, parts of the culture were shifted to 37 °C and competence development in these cultures was followed during 90 min (×---×). The time of exposure to DNA was 15 min.

Fig. 7 (b). Response of 3G-18 to a temperature shift to 37 °C as a function of growth time at 42 °C. The conditions in this experiment were the same as described for Fig. 7 (a). ○—○, Competence development during growth at 42 °C; ×---×, competence development at 37 °C.

Fig. 6*a, b* shows the effect on growth and competence development of shifting a Com^{ts}M8 culture from the restrictive temperature at the time when the interruption in its growth curve had occurred to the permissive temperature. It can be seen that the culture responds with reinitiation of growth and an explosive development of competence. When the culture is shifted back to the restrictive temperature during its shift-induced development of competence further progress of competence development is prevented; only a relatively poor wave of competence is observed. If the culture is shifted back at the time when the peak of competence has been reached, a rapid loss of competence is observed. The parental strain, different from the mutant in being able to develop competence when cultured continuously at 42 °C, behaves in exactly the same way, responding with very rapid development of competence when shifted from 42 to 37 °C, displaying only a poor wave of competence when shifted from 37 to 42 °C during its development of competence, and rapidly losing competence when shifted from 37 to 42 °C at the time when maximal competence is achieved (Fig. 6*c, 7b*). Since the mutant culture after being shifted back to the restrictive temperature develops competence characteristic for the parental strain after such a shift, it follows that the relatively short stay at the permissive temperature (56 min) suffices the mutant to obtain the wild-type phenotype at the restrictive temperature. This also applies to the postexponential growth behaviour of the mutant: as can be seen from Fig. 6*a*, shifting back the mutant to the restrictive temperature results in the continuation of the shift-induced resumption of growth.

The rapid manifestation of competence in the Com^{ts}M8 bacteria following a shift from 42 to 37 °C strongly suggests that the mutant is blocked in a relatively late step in its development towards competence. To establish whether the mutant persists in this (precompetent) state at 42 °C during the full length of the incubation period, or whether this state is transiently acquired, the capacity of cultures growing at 42 °C to develop competence during a fixed period of time following a shift to 37 °C was followed as a function of growth time. For comparison, the parental strain was treated similarly. Fig. 7*a* shows that the number of transformable cells produced by the mutant during a fixed period of exposure to 37 °C is decreasing as a function of time of growth at 42 °C, indicating that the precompetent state is transiently acquired by the mutant culture at 42 °C. Fig. 7*b* shows that during the whole period of competence development at 42 °C competence in the parental strain is enhanced by a shift to 37 °C, indicating that in the parental strain also, part of the precompetent fraction cannot pass on to the competent state at 42 °C. Even after the parental culture has passed its period of competence at 42 °C, the appearance of new competent cells can still be induced by transfer to 37 °C. In both the mutant and the parental strain the presence of a temperature-inducible competence potency seems to be transient. These results suggest that the characteristic difference between the parental and the mutant strain consists of the complete inability of the precompetent mutant bacteria to pass into the competent state at 42 °C, whereas the parental strain is partly able to do so.

DISCUSSION

The results of experiments to characterize the Com^{ts} mutant indicate that the mutant is unable to become transformed at the restrictive temperature because it is unable to take up exogenous DNA. The mutation causing this defect is pleiotropic: under the non-permissive conditions an aberration in the post-exponential growth phase also occurs. This implies that at least one step in the development of competence is dependent on a gene product that simultaneously controls post-exponential growth.

Shifting the mutant culture from the non-permissive to the permissive temperature and vice versa rapidly results in a change of competence development identical to that observed in the parental strain after such shifts. Furthermore, a relatively short time of exposure to the permissive temperature after growth at the restrictive temperature enables the mutant culture to acquire its wild-type phenotype at the restrictive temperature (Fig. 6).

Recent studies by Dooley *et al.* (1971) have led to the recognition of the precompetent state through which a cell must pass before the acquisition of the transformable state. Since they found that the precompetent phase precedes the competent state by 90 to 180 min, the rapid appearance of transformable mutant bacteria upon shifting the temperature from the restrictive to the permissive one (Fig. 6*b*) indicates that the bacteria have already developed precompetence at the time of the shift. Although able to develop a fair degree of competence when continuously cultured at 42 °C, the parental strain also rapidly responds to a shift in temperature to 37 °C, inferring that in 42 °C cultures many of the parental bacteria also fail to pass from the precompetent to the competent state. Since the precompetent state seems to be transiently acquired at this temperature, it is concluded that the transience of the competent state is governed by that of the precompetent phase. The characteristic difference between the two strains apparently consists of the partial ability of the precompetent bacteria of the parental strain to pass on to the competent state at 42 °C, whereas the mutant precompetent bacteria are completely unable to do so.

The inability of the mutant to take up exogenous DNA and its changed cell morphology

observed at the restrictive temperature (Fig. 5) suggest that the mutant has an altered cell envelope. However, a significant difference between the two strains in their sensitivity to deoxycholate, EDTA, lysozyme or osmotic shock could not be established; in addition electron-microscopic examination of bacteria at various times during growth did not reveal distinct differences in their general appearance or in their components.

Recently we reported that *Bacillus subtilis* cultures excrete competence-stimulating activity into the culture fluids; this activity causes an earlier initiation of competence development when physiologically low-competent cultures are exposed to such culture fluids (Joenje *et al.* 1972). Mutant Com⁺M8 was found to produce competence stimulating activity at 42 °C (unpublished results) but is apparently not able to respond to it.

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