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ABSTRACT

Forty-one temperature sensitive mutants selected on nutrient agar were able to grow at the non-permissive temperature when the osmotic concentration of the medium was increased. These mutants were classified according to (a) growth patterns at 42C on various complex media, and (b) differential sensitivity to three antibiotics. Three salts and three carbohydrates were tested as osmotically active agents for their ability to relieve the temperature sensitivity; the salts were much more effective than the carbohydrates. Osmolality profiles at 42C with NaCl showed that three mutants would not grow at either extreme of osmolality while one was inhibited at low osmolalities only. Cells shifted to 42C after growth at 30C in a medium of low osmolality elongated without dividing, yet retained the ability to divide at 30C. It is suggested that the cell membrane is defective, affecting permeability at both temperatures and cell division at the non-permissive temperature. A higher osmotic environment appears to stabilize the defect, allowing growth at the non-permissive temperature.

RESUME

Quarante et un mutants sensibles à la température, ont été sélectionnés sur un milieu nutritif gélosé, où ils se sont montrés capables de croître à une température qui ne permet pas habituellement une croissance lorsque la pression osmotique est augmentée. Ces mutants ont été classés suivant: (a) leurs possibilités de croissance à 42C dans divers milieux plus ou moins complexes, et (b) leurs sensibilités à trois antibiotiques différents. On a également testé trois sels et trois carbohydrates capables de supprimer la sensibilité à la température. Les sels se sont montrés plus efficaces que les carbohydrates. La courbe de l'osmolalité à 42C avec NaCl a montré que trois mutants ne pouvaient pas croître aux extrêmes osmolalités alors qu'un autre était seulement inhibé aux basses osmolalités. Les cellules incubées à 30C dans un milieu à basse osmolalité ont été placées à 42C où elles peuvent s'allonger sans toutefois se diviser; replacées à 30C ces mêmes cellules sont capables de se diviser à nouveau. On pense que la membrane cellulaire est défectueuse, sa perméabilité est modifiée par la température, et la division cellulaire seulement à la température qui ne permet pas la croissance. Un environnement osmotique élevé semble faire disparaître la défectuosité, ce qui permet la croissance à la température non-permissive.

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Forgive, O Lord, my little jokes on thee,

And I'll forgive thy great big one on me.

Robert Frost

INTRODUCTION

Temperature sensitivity in microorganisms has been a useful genetic tool for at least twenty-five years. Its discovery, however, was accidental. In 1946, Mitchell and Houlahan were studying adenine auxotrophs of Neurospora crassa and found, surprisingly, that three of them were temperature dependent. These three mutants were not able to synthesize adenine at temperatures above 33C, although they could at temperatures from 10C to 26C. In 1950, Horowitz summarized what was then known about "temperature mutants" of Neurospora, some of which were sensitive to higher temperatures, others to lower temperatures. Many of these were nutritional auxotrophs at the non-permissive temperature. At this time there was still much discussion about Beadle and Tatum's "one gene-one enzyme" hypothesis and the results from studies on temperature mutants such as these were used to support the theory. For example, Horowitz and Leupold (1951) were the first to isolate temperature sensitive (ts) mutants of E. coli K12. They found that almost 75% of their 161 ts mutants were able to grow at 40C on minimal medium supplemented with various amino acids or vitamins, i.e. they were defective in one step of a particular biochemical pathway.

The other 25% were defective in "indispensable functions", i.e. the mutation could not be overcome by simply adding one amino acid or vitamin to the medium.

To explain a mutation to temperature sensitivity, several possibilities were proposed. The first was that the mutant produced an enzyme that was more heat labile than that of the wild-type. The second was that the gene itself was non-functional at the higher temperature. The third, and least likely, was that an inhibitor was produced at the higher temperature. All three possibilities assumed that the mutation to temperature sensitivity resided in one gene that controlled one enzyme. The presence of a heat labile enzyme was the first possibility to be explored. McElroy and Mitchell (1946) looked for a ts adenosine deaminase in one of the ts adenine auxotrophs of Neurospora previously mentioned. They did not find it. It is possible that they were studying the wrong enzyme. On the other hand, Maas and Davis (1952) reported a definitely heat labile pantothenate-synthesizing enzyme from a ts mutant of E. coli that required pantothenate at temperatures above 30C. Their enzyme preparation was relatively pure and they found that sucrose and KCl had a protective effect. They concluded that the defect was in the enzyme molecule itself and ruled out the possibility of an inhibitor. The use of temperature sensitive mutants to study various functions of the cell was thus launched.

Since then, a large body of literature has accumulated on ts mutants. They have been produced in bacteriophages (Streisinger et al. 1961; Edgar and Epstein 1965; Delgano and Sinsheimer 1968) and even in mouse L-cells (Thompson et al. 1970). A cold sensitive mutant

of Drosophila has also been reported (Suzuki 1970). However the following discussion will be confined to bacteria and yeasts. An attempt will be made to discuss temperature sensitivity as it affects the following vital functions of the cell: (a) protein synthesis; (b) the structure of the cell envelope; and (c) DNA synthesis. I will then discuss suppression that either causes or relieves temperature sensitivity.

(a) Protein Synthesis

The mechanism of protein synthesis is now well understood, particularly in E. coli, and will not be discussed here. Mutants temperature sensitive for protein synthesis have been isolated (Kohiyama et al. 1966; Boyd et al. 1968; Russell and Pittard 1971a). In some cases a particular enzyme has been found to be defective; in others, no simple explanation can be found.

The most common defect in temperature sensitive protein synthesis mutants appears to be the presence of ts tRNA synthetases. Böck (1968) reported ts phenylalanyl tRNA synthetases in E. coli which had weakened subunit interactions. This would cause thermolability and would stop protein synthesis at the non-permissive temperature. Low et al. (1971) reported two ts mutants of E. coli, one with an altered leucyl-tRNA synthetase, the other with an altered seryl-tRNA synthetase. In both cases the mutant enzymes were thermolabile. Yaniv (1968)

found altered valyl-tRNA synthetases in a ts mutant of E. coli. Recently, Atherly and Suchanek (1971) found that the temperature sensitivity of their protein synthesis mutants of E. coli K12 was caused by inactivation of phenylalanyl-tRNA synthetase.

A ts tRNA synthetase can also exhibit faulty charging activity causing mistranslation. Steinberg and Anagnostopoulos (1971) found a ts tryptophanyl-tRNA synthetase in a ts mutant of B. subtilis. They concluded that at least part of the defect was in the amino acid recognition step. Russell and Pittard (1971a) found several ts synthetases that were altered in both the amino acid activation and the tRNA-charging steps. A similar defect was found in a ts mutant of Neurospora crassa where an altered leucyl-tRNA synthetase caused mistranslation (Printz and Gross 1967).

However, not all ts protein synthesis mutants can be explained this way. One alternative is a ts enzyme affecting one specific pathway, such as the thermolabile homoserine transsuccinylase found by Ron and Davis (1971) in E. coli. Another interesting possibility is that reported by Khesin et al. (1968). They found low levels of RNA polymerase in a mutant of E. coli ts for protein synthesis. More interesting still are the reports of temperature sensitive regulation. McFall and Heincz (1970) found a mutant of E. coli K12 with thermosensitive regulation of D-serine deaminase synthesis. They concluded that this synthesis was under negative control and that their mutant possessed a thermolabile repressor. Irr and Englesberg (1971) reported ts regulatory mutants for the L-arabinose operon in E. coli B/r. They concluded that both repressor and activator activities were thermolabile.

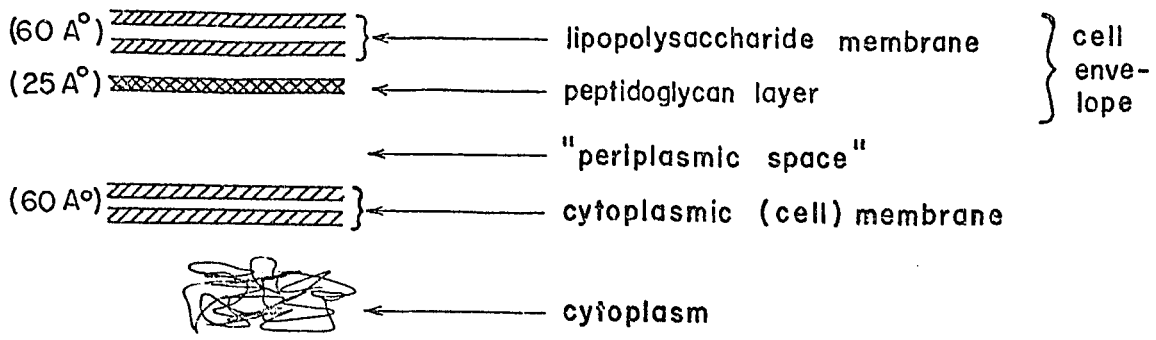
Another possibility, and apparently the most difficult to determine, is a defect in the ribosomes. Kang (1970) found a ts streptomycin protein in E. coli which stopped protein synthesis at the higher temperature. Kanamitsu et al. (1970) reported a ts mutant of B. subtilis that appeared to have temperature sensitive ribosomes. When treated with heat, the ribosomes of the mutant dissociated to a greater extent than did those of the parent. They could not say, however, whether the defect was in the RNA or in one of the ribosomal proteins.

All these mutants produce temperature sensitive proteins; the differences are the levels at which they affect the cell.

(b) The Cell Envelope

The cell wall gives shape and rigidity to the bacterial cell. In gram positive organisms, the wall is made up of a thick peptidoglycan layer. In gram negative organisms, the peptidoglycan or wall layer is relatively thin with a complex outer layer of lipopolysaccharide and protein attached to it. It is this outer layer that is largely responsible for the staining differences. In gram negative bacteria, the cell wall layer together with the outer LPS layer is often referred to as the "cell envelope".

The following is a model of part of the cell envelope of E. coli (adapted from dePetris 1967). 'Peptidoglycan' refers to a structural unit made up of two sugar derivatives, N-acetylglucosamine



and N-acetylmuramic acid, and a small group of amino acids: D-alanine, L-alanine, D-glutamic acid and either lysine or diaminopimelic acid. Repeating units of peptidoglycan are cross-linked to form a three-dimensional cell wall. According to Forsberg et al. (1970), the lipopolysaccharide membrane is a double-track layer, the outer track being lipoprotein and the inner track being lipopolysaccharide.

The cell periphery is not only structurally but also functionally complex. Several enzymes are thought to be localized near the surface of the cell in the "periplasmic space" (Brockman and Heppel 1968). The cell membrane is the site of several transport systems, for both cations and small molecules (Fox and Kennedy 1965; Rogers 1968; Bhattacharyya 1970). These functions allow the bacteria to adapt to changes in their environment. Obviously a defect anywhere in this complex structure would have severe consequences for the cell.

Mutants with envelope defects have indeed been isolated. A mutant of the envA gene in E. coli K12 showed increased uptake of gentian violet and increased sensitivity to lysozyme (Normark and Westling 1971). This mutant also had less phosphatidylglycerol in

the cell envelope than did the parent. There appeared to be some kind of coupling between protein and lipid synthesis, similar to the coupling between RNA and protein synthesis. Obviously an alteration in the lipid content of the cell envelope could cause increased penetrability, primarily due to its function as a nonspecific permeability barrier (Buller and Dobbs 1971).

Recently, Holland et al. (1970) described mutants of E. coli K12 with a membrane defect. A mutation in the cet gene caused some of the mutants to become insensitive to colicin E_2, to show reduced replication of phage lambda and to have defective division. They suggested an alteration in the specific membrane component for the site of binding of colicin E_2. They also implicated this site in cell division. This is logical, since it is now accepted that there is a site on the cell membrane where DNA replication takes place (Jacob et al. 1966). Similarly, the product of the tolC locus in E. coli K12 appears to be involved in membrane structure and function (Whitney 1971). A mutation in this gene confers tolerance to colicin E_1 only, the mutant cells retaining sensitivity to the other three colicins. Mutations in this gene cause pleiotropic effects similar to those caused by the cet gene.

A mutation in the env gene alters the cell envelope, with concomitant changes in nucleic acid synthesis; a mutation in the cet or tol genes alters membrane structure, causing altered permeability and/or defective division. These defects emphasize the importance of envelope and membrane structure, since one such mutation always affects other vital functions of the cell.

Mutants with temperature sensitive defects in the cell membrane have also been isolated (Kohiyama et al. 1966). Inouye and Guthrie (1969) described an alteration in one protein of the cell membrane in a ts mutant of E. coli. This mutant was unable to synthesize DNA at 41C but could continue cell division at that temperature. They attributed the temperature sensitivity of DNA synthesis in this mutant to this particular ts protein component of the membrane. Similarly, Siccardi et al. (1971) found E. coli mutants ts for DNA replication which had alterations in the membrane. Two loci, DnaA and DnaB, were involved, the former affecting one particular protein component of the membrane (possibly the same protein as that reported by Inouye and Guthrie), the latter affecting how the membrane proteins are held together. Although these groups reported mutants ts for DNA synthesis, the primary defect in both cases appears to be in the cell membrane.

Weigand et al. (1970) reported a different type of ts membrane mutation in E. coli. Upon shifting to the non-permissive temperature, the mutant increased in size and acquired "extra membranes", i.e. membranous structures appearing as vesicles or whorls in the cytoplasm. However, they could not determine whether the ts defect was associated with membrane production, cell division or a metabolic process within the cell. Possibly DNA replication is temperature sensitive while membrane synthesis continues. Kohiyama et al. (1966) reported ts mutants for cell division that possibly had altered membranes. They suggested that either the mesosomes or septum formation were affected, thus inhibiting cell division.

Still another ts membrane mutation was described by Crandall

and Koch (1971), also in E. coli. Their mutants were temperature sensitive for β-galactoside transport but also showed decreased generalized transport. They found that the permease of the mutant was not heat labile but rather its synthesis was decreased at the high temperature. They attributed this temperature sensitive synthesis to a membrane component at the binding site for the permease. Since these mutants showed generally increased permeability, the membrane must certainly be altered.

Defects in the cell wall (peptidoglycan layer) have been implicated in ts division mutants of E. coli (Reeve et al. 1970). Apparently the mutation affects a late step in cell wall synthesis, without which cell division cannot occur. Kohiyama et al. (1966) reported two ts mutants of E. coli K12 that lysed upon shifting to 40C. Apparently these cells are defective in the synthesis of diaminopimelic acid.

These are just a few examples of membrane and envelope mutations as primary defects. Alterations in lipid biosynthesis could also cause such defects (Normark 1971). As previously mentioned, almost all these temperature sensitive mutations are due to altered enzymes stopping or slowing down the metabolic machinery of the cell.

(c) DNA Synthesis

DNA replication and the subsequent separation of the cells is a complex process that is not well understood. Many DNA synthesis

mutants have been reported, some of which have already been discussed (Siccardi et al. 1971; Inouye and Guthrie 1969). The following discussion will deal with mutations in the actual process of DNA replication and the resulting alterations in cell division.

In 1958, Kornberg and his coworkers isolated a DNA polymerase which was then thought to be the polymerase required for DNA replication (Lehman et al. 1958). It is now thought that this is a repair enzyme (deLucia and Cairns 1969). Fangman and Russel (1971) reported mutants of E. coli that were temperature sensitive for both DNA replication and the repair of single-strand chromosome breaks (X-ray induced). They postulated that a ligase necessary for both these processes was defective. Marinus and Adelberg (1970) also reported ts DNA synthesis mutants in E. coli K12. However in this case the defect appeared to be due to two thermolabile gene products. Recently, Karamata and Gross (1970) have suggested that a minimum of 14 genes are involved in DNA replication in B. subtilis, the actual number probably being thirty.

DNA synthesis mutants usually form long filaments similar to the "snakes" produced in normal cells after growth in nalidixic acid, an inhibitor of DNA synthesis (Kohiyama et al. 1966). Using synchronous cultures of E. coli B/r, Clark (1968) found that DNA synthesis is required for cell division to occur. He suggested that the end of a round of replication somehow commits the cell to divide. Generally, cell division in E. coli occurs about 20 minutes after the replication round has been completed (Cooper and Helmstetter 1968). Walker and

Smith (1970) isolated conditional cell division mutants in E. coli which they called lon⁻. The lon gene specifically inhibits septum formation, resulting in long multinucleate filaments. Such mutants have a decreased DNA/mass ratio due to a decreased rate of DNA synthesis. These authors also suggested that lon⁻ mutants are unable to synthesize some kind of division activator, thus uncoupling DNA synthesis and cell division. On the other hand, Hirota et al. (1968) reported mutants of E. coli K12 ts for DNA synthesis that could still form septa at the non-permissive temperature. They concluded that two mutations were involved. The first, called T46, caused the temperature sensitivity, stopping both DNA synthesis and septum formation; the second, called DivA, would allow septum formation and cell division but only when DNA synthesis was stopped by the T46 mutation. In other words, simultaneous mutations in both these genes would produce DNA-less cells. The DivA locus may code for a division activator as postulated by Walker and Smith.

A different type of ts DNA synthesis mutant in E. coli was reported by Worcel (1970). Upon shifting to the non-permissive temperature, the mutant immediately stopped DNA synthesis. A down-shift back to the permissive temperature allowed re-initiations in one of the two daughter chromosomes. The ts defect appeared to be in the actual growth of the DNA chain, not in the initiation process.

Mutants of E. coli ts for DNA initiation have been reported. Shapiro et al. (1970) isolated ts mutants with defective cell division. Since cell division is controlled by DNA synthesis, they found that

these mutants were actually unable to initiate DNA synthesis at the non-permissive temperature. They presented two possible explanations: (i) an enzyme directly involved in initiation is altered so that at the higher temperature it is unstable and cannot fit into the membrane, which is secondarily altered; (ii) the membrane itself contains a defective protein which does not allow the insertion of initiation enzymes at the non-permissive temperature. There is insufficient evidence to choose between these two alternatives. Carl (1970), also using E. coli, isolated ts mutants, some with residual DNA synthesis at 40C, others immediately stopping DNA synthesis at 40C. He placed these mutants in two groups according to their ability to support growth of phage T4 and/or phage lambda at 41C. The two mutations mapped at different loci. He suggested that the defect was in initiation of DNA synthesis but again there is no substantial evidence for this conclusion.

Carl made an interesting observation in this report. One of his mutants was "salt-rescuable", i.e. the addition of 2% NaCl to the medium overcame the temperature sensitivity of the cells. This effect of salt on ts DNA synthesis mutants was first noted by Ricard and Hirota (1969). The importance of this observation will be discussed further in the following section.

The previous discussion strongly suggests the existence of a complex, well co-ordinated system for bacterial reproduction. The difficulties in pinpointing a mutation anywhere in this process are obvious. It is conceivable that Karamata's estimate of 30 genes for

DNA replication is only a part of the total required for reproduction in these organisms.

Suppression

The term 'suppression' generally refers to a mutation in one cistron that somehow overcomes or "suppresses" a mutation in a different cistron. The existence of suppressor genes has been known for quite some time (for a review, see Gorini and Beckwith 1966). Suppression can also be effected by an external agent, such as that observed in some cases by streptomycin, and is generally referred to as "phenotypic suppression". Temperature sensitivity is generally caused by a missense mutation, resulting in the production of an altered protein. Since suppressors are believed to alter either tRNA specificity or some other component of the protein synthesizing machinery, the finding of suppressors that either cause or relieve a ts mutation is not surprising.

In 1966, Apirion reported a suppressor causing temperature sensitive growth in E. coli K12. He induced a mutation in a tryptophan auxotroph that simultaneously suppressed the tryptophan requirement at 30C while causing temperature sensitivity at 43C. He found that the 50S subunit of the ribosome was more heat sensitive in this strain than in the parent. He concluded that the mutation caused a change in a component of the 50S subunit such that translational

mistakes occurred at both temperatures; the mistakes at 30C suppressed the tryptophan requirement while at 43C the mistakes were too extensive to allow growth. Eggertsson (1968) reported a different suppressor, supH, that also caused temperature sensitivity in E. coli. Although there is no direct evidence, this locus was thought to affect a specific tRNA, causing mistranslation. A similar situation, also in E. coli K12, was recently reported by Gallucci et al. (1970). They induced a mutation in the su-4 gene, producing a temperature sensitive tyrosine tRNA. The su-4 gene is a suppressor gene and this mutation caused "temperature dependent suppression".

The opposite type of suppressor was recently reported by Russell and Pittard (1971b). Their strain of E. coli K12 was temperature sensitive due to a mutation in the pheS gene. An extragenic suppressor, supQ, restored the cells' ability to grow at the non-permissive temperature. However, these authors could not determine if this locus functioned at the level of translation.

Generally, a suppressor gene produces an altered tRNA. What happens in the case of "phenotypic suppression"? As previously mentioned, streptomycin can suppress some mutations. It is thought that this antibiotic causes misreading, i.e. the effect is at the ribosomal level. There is one other case of phenotypic suppression that is of interest here, that of "osmotic suppression".

In 1964, Hawthorne and Friis reported a new class of mutants in yeast which they called "osmotic remedial mutants". These were auxotrophic mutants whose nutritional requirement was abolished by

increasing the osmotic concentration of the medium. There have been several reports of this osmotic phenomenon overcoming temperature sensitivity. Kuwana (1961) reported temperature sensitive mutants of Neurospora crassa which would grow at the non-permissive temperature only when an osmotic agent was present. He concluded that a defect in either the cell membrane or in a transport system was responsible, this defect being stabilized by a higher osmotic concentration in the medium. Similarly, Wainwright and McVeigh (1967) reported ts mutants of Neurospora crassa with an osmotic requirement for growth at the higher temperature. They could not determine if their mutants had a transport defect or if some macromolecular component of the cell was heat labile. Recently, Good and Pattee (1970) reported ts "osmotically fragile mutants" in Staph. aureus which they thought had defective cell walls. However, the validity of this report remains to be established.

The most interesting report on this subject, however, is that of Ricard and Hirota (1969). Their mutants of E. coli were temperature sensitive for DNA synthesis. By adding various salts or sugars to the medium, these mutants became phenotypically normal at the non-permissive temperature. They concluded that this osmotic effect was actually on the cell membrane, but the evidence is inconclusive.

Since temperature sensitivity is usually caused by a missense mutation producing an altered protein, the only logical conclusion is that the higher osmotic concentration somehow stabilizes

this protein at the non-permissive temperature. A defective membrane protein would be the first possibility, since a defect in the membrane could cause multiple effects, e.g. leakage, faulty DNA synthesis, faulty cell division. In the cases cited, the exact defects are unknown. The work to be described here only adds to the present confusion.

STATEMENT OF PROBLEM

The original problem was to test if S-adenosylmethionine (SAM) was the active principle for chemotaxis in *E. coli*. Since a mutation affecting the synthesis or use of SAM would be lethal, we selected for conditionally lethal mutants, specifically temperature sensitive mutants. Hopefully such mutants would be non-chemotactic only at the non-permissive temperature. During this investigation, it was found that many of the ts mutants grew on semi-solid tryptone plates at 42C but did not grow on nutrient agar at the same temperature. The main difference in the two kinds of plates was the presence of 0.5% NaCl in the soft plates. When 0.5% NaCl was added to nutrient agar, the mutants grew at 42C; no growth was obtained at 42C on tryptone plates without added NaCl. Since the salt was somehow alleviating the temperature sensitivity in many cases, it was decided to investigate this phenomenon further. When the addition of other salts or sucrose also permitted growth at 42C, the phenomenon was tentatively called "osmotic suppression of temperature sensitivity". The following experiments were done to find some explanation for this phenomenon.

MATERIALS AND METHODS

(a) Media

Tryptone, peptone, yeast extract and casamino acids were used as 1% solutions. Nutrient agar and penassay were made up in the concentrations recommended by Difco. Semi-solid tryptone plates contained 0.35% agar; all other plates contained 1.5% agar. Top agar, as used in an agar overlay, contained 0.7% agar. Tris maleate buffer contained 6.0 gm Tris base, 5.8 gm maleic acid and 1.0 gm $MgSO_4.7H_2O$ per litre; the pH was adjusted to 6.0 with NaOH. Minimal medium contained 11.2 gm K_2HPO_4, 4.8 gm KH_2PO_4, 2.0 gm $(NH_4)_2SO_4$, 1.0 ml 1M $MgSO_4$ and 1.0 ml of 50 mg/100 ml $Fe_2(SO_4)_3$ in 870 ml; 100 ml of 10% glucose; 10 ml each of filter sterilized 1% threonine, 1% leucine and 1% histidine; and 1.0 ml of 1 mg/ml vitamin B_1. H1 buffer contained only the salts of minimal medium.

(b) Bacteria

AW405, a strain of E. coli K12, requires histidine, leucine,

threonine and vitamin B_1 for growth. This strain was used to select for cells that were extremely motile at 42C. A loopful of the bacteria was placed in the centre of a semi-solid tryptone plate which was then incubated at 42C. After the cells had swarmed out from the inoculum, a loopful was picked from the edge of the outermost ring and placed in the centre of a second soft plate. The entire procedure was repeated several times until a very motile culture was obtained. This 'strain', called A014, was used as the parent for mutagenesis.

(c) Mutagenesis

An exponentially growing culture was mutagenized with N-methyl-N' nitro-N-nitrosoguanidine as follows. Five ml of culture was filtered through a millipore filter and washed with 10 ml of Tris maleate buffer. The membrane was placed in a fresh flask with 5.0 ml of buffer, agitated on a vortex, then removed. NTG, 0.5 ml of a 1 mg/ml solution, was then added and the culture incubated at 37C. After 30 minutes, 1.0 ml was filtered and washed with 10 ml of H1 buffer. The cells were resuspended in 10 ml of minimal medium as above. Several 0.1 ml aliquots of a 10^2 dilution were plated on nutrient agar and incubated at 30C. After growth, colonies were replica plated onto nutrient agar at 42C. Colonies that failed to grow at 42C were picked and grown in a small volume of tryptone broth, then retested for growth at 42C. This was repeated several times to eliminate revertants.

(d) Measurement of Osmolality

Osmolality was measured in an Advanced Osmometer (Advanced Instruments, Inc., Newton Highlands, Massachusetts). This instrument utilizes the freezing point depression method for measuring osmolality.

When different compounds were tested, a concentrated solution was made up in tryptone broth, then added to tryptone broth in varying amounts to obtain a standard curve of osmolality for each. Figure 1 shows the standard curves obtained for NaCl, KCl and $MgCl_2$. The osmolality of tryptone broth was approximately 60 mOsm.

(e) Test for Antibiotic Sensitivity

Two types of plates were used, tryptone and tryptone plus 0.5% NaCl. A bottom layer of 10 ml was covered with an overlay of 4 ml containing 0.2 ml of an overnight culture. After the overlay had hardened, eight sensi-discs (Baltimore Biological Laboratory, Division of B-D Laboratories, Inc.) containing different antibiotics were simultaneously placed on top using a sensi-disc dispenser. Each mutant was tested at both temperatures, the tryptone plates at 30C and those with NaCl at 42C.

(f) Shift Experiments

Cultures were incubated at 30C in 15 ml of tryptone broth

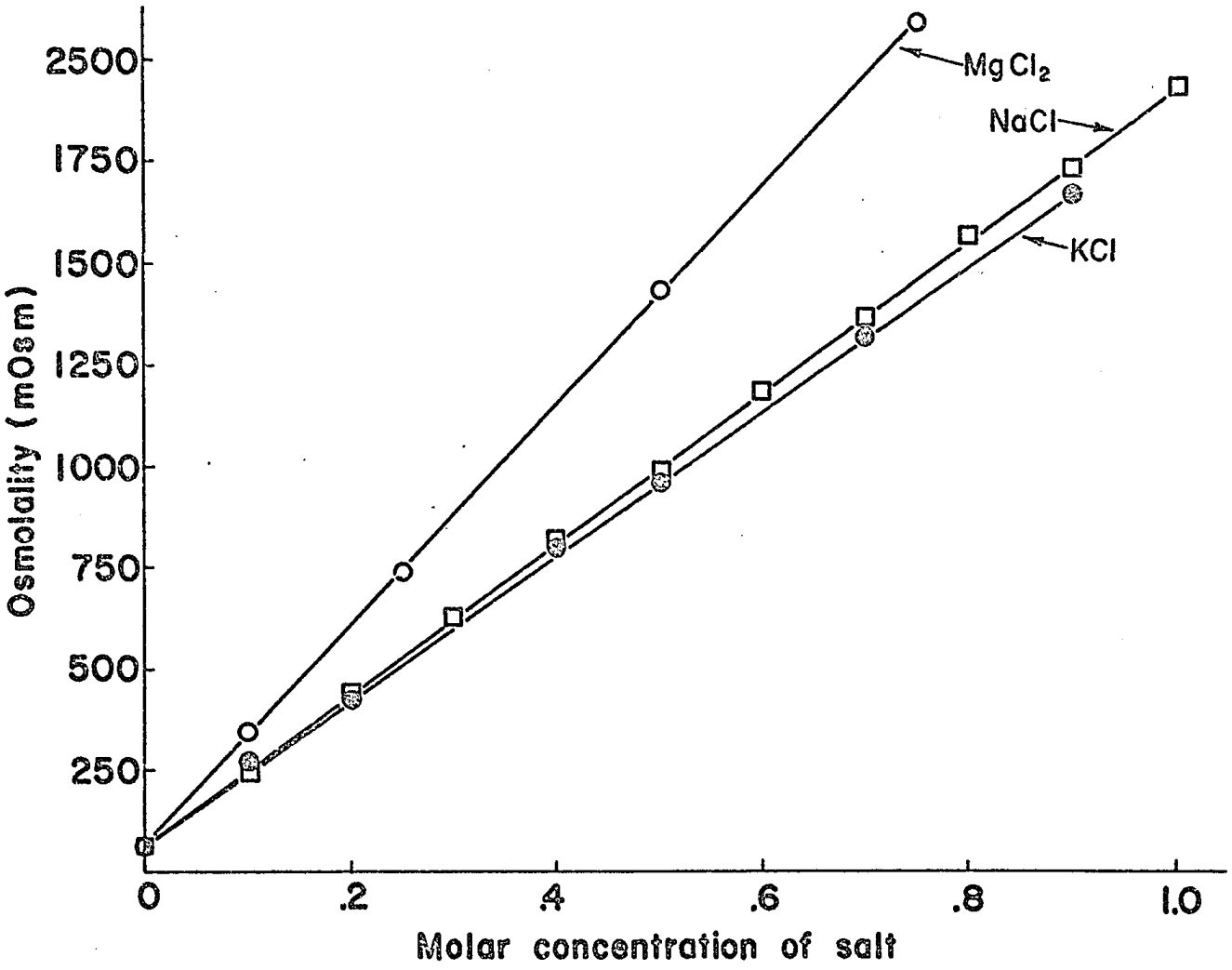

2500
1750
1500
1250
1000
750
500
250
Osmolality (mOsm)
0
.2
.4
.6
.8
1.0
Molar concentration of salt
MgCl2
NaCl
KCl

Figure 1 - Standard curves of osmolality versus concentration for NaCl, KCl and $MgCl_2$. A 1M solution of each was made up in tryptone broth, then diluted with tryptone broth to give the concentrations tested.

in 18 mm side-arm flasks. Optical density was measured at 650nm in a Coleman Junior II spectrophotometer (model 6/20). At the desired O.D. (see results) the flasks were placed at 42C and O.D. readings taken at 3-hour intervals. In some cases the cells were shifted back to 30C after different periods of time at 42C.

When salt was added to cells at 42C, 5.0 ml of a 1M solution of NaCl was added to each flask to give a final osmolality of approximately 500 mOsm.

RESULTS

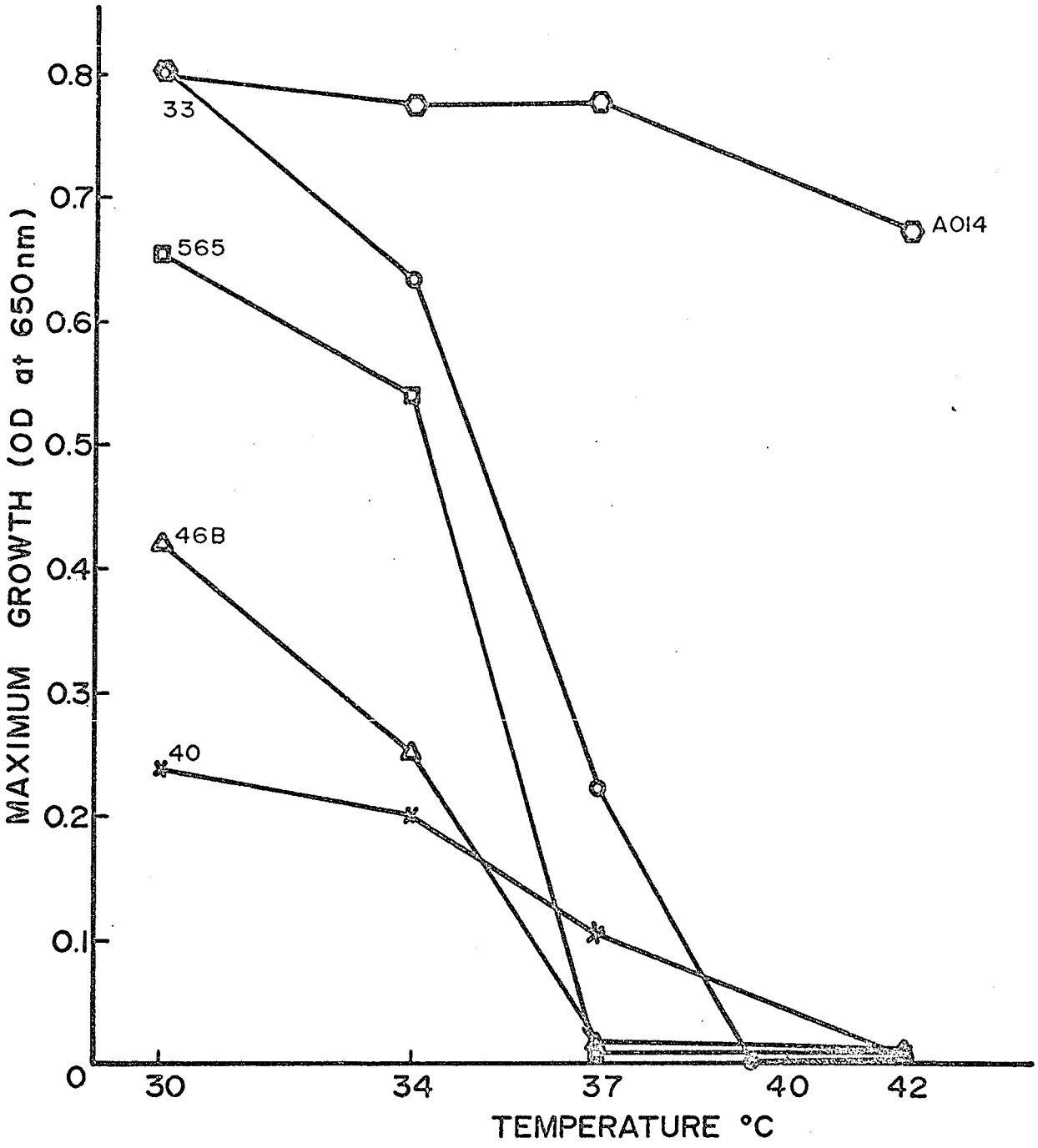
Sixty-eight temperature sensitive mutants were obtained but many proved difficult to work with because of high reversion rates. The temperature sensitivity of several of the more stable mutants was examined in liquid medium (Figure 2). All the mutants grew best at 30C but growth rate and maximum growth varied considerably. None grew at temperatures above 37C. The results were essentially the same whether growth rate or maximum growth was determined. The latter was used in the majority of the experiments reported below, since it was the more convenient parameter to determine.

CLASSIFICATION

(a) Osmotic Effects

When several mutants were tested on semi-solid tryptone plates for motility as well as on nutrient agar, growth was obtained at 42C. Since both media are nutritionally complete, the only other

Figure 2 - Temperature profiles of mutants 33, 565, 46B and 40 compared to the parent (A014). Each mutant was tested for growth in tryptone broth.


MAXIMUM GROWTH (OD at 650nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
33
A014
565
46B
40
30
34
37
40
42
TEMPERATURE °C

difference being the presence of 0.5% NaCl in the tryptone plates, further tests were carried out to see whether this was an osmotic or an ionic effect.

Preliminary tests were done on tryptone plates containing either 0.5% NH_4Cl or KCl, or 0.2M (6.5%) sucrose. The presence of any one of these compounds allowed growth at the non-permissive temperature. It was concluded that this was indeed an osmotic effect.

The ability of the mutants to grow on plates of various complex media was also tested. Seven different kinds of media were used, and 0.5% NaCl was added to increase the osmotic concentration in three cases. All mutants were tested at both 30C and 42C. Table 1 shows 7 different patterns of response at 42C. Since all these media are complex, the different patterns obtained were attributed to the differences in osmolality. Group VII is an odd exception. These mutants did not grow on penassay although they grew on media of lower osmolality. Possibly something in this medium inhibited their growth at the non-permissive temperature.

The mutants were also tested on minimal medium, minimal medium + 0.5% NaCl, and minimal medium + NaCl + 0.1% yeast extract. The osmolalities of these media were 309 mOsm, 460 mOsm and 465 mOsm respectively. Some mutants did not grow on minimal medium at 30C, indicating a nutritional requirement. Many mutants grew poorly on minimal medium at 30C and either grew poorly or not at all at 42C. Four of the six Group I mutants, which did not grow at 42C on any of the media listed in Table 1, did grow on minimal medium supplemented with salt and yeast extract at 42C, indicating a high osmotic requirement. The

TABLE 1

Growth of temperature-sensitive mutants on various complex media at 42C

group	(no.)	peptone nutrient agar tryptone	yeast extract	casamino acids	casamino acids + 0.1% yeast extract	+ 0.5% NaCl peptone tryptone nutrient agar	penassay
		<60 mOsm[a]	76	132	140	204 - 228	238
I	(6)	-[b]	-	-	-	-	-
II	(2)	-	-	-	-	-	++[c]
III	(10)	-	-	-	-	++	++
IV	(5)	-	-	-	++	++	++
V	(8)	-	-	++	++	++	++
VI	(3)	-	++	++	++	++	++
VII	(7)	-	-	-	-	±[d]	-
total:	41						

a - osmolality of the liquid medium from which the plates were prepared

b - - indicates no growth

c - ++ indicates growth

d - ± indicates growth on one or two of these media but not on the third

other two mutants of this group grew poorly on this medium at 30C and not at all at 42C, allowing the possibility of both a high nutritional and a high osmotic requirement.

A total of forty-one mutants was retained after this classification. The remaining twenty-seven mutants showed high reversion rates and were discarded.

(b) Antibiotic Sensitivity

When several mutants were examined under the microscope after growth at different temperatures, variations from the normal "short-rod" morphology were observed. Such differences are typical of septum and other envelope mutants. Since envelope mutants often show differential sensitivity to antibiotics, all mutants were tested at both 30C and 42C against ten different antibiotics.

All mutants and AO14 were sensitive to chloramphenicol, ampicillin, nalidixic acid and kanamycin at both temperatures. None were sensitive to valinomycin, lincomycin or streptomycin at either temperature, nor was AO14. The mutants showed altered sensitivities to three antibiotics: vancomycin, bacitracin and novobiocin as seen in Table 2. No mutant showed a change from the parent at 30C only. Six mutants became sensitive to one or more antibiotic at 42C only. The majority became sensitive to one or more of these antibiotics at both temperatures.

TABLE 2

Patterns of response to three antibiotics at both temperatures

	VANCOMYCIN		BACITRACIN		NOVOBIOCIN		number of mutants	
	30C	42C	30C	42C	30C	42C		
wild-type	s	-	-	-	-	-	1	
A	s	s	-	-	-	-	2	
	s	-	-	-	-	s	2	
	s	s	-	-	-	s	1	
	s	s	-	s	-	s	1	
								6
B	s	-	s	-	-	s	3	
	s	-	-	-	s	s	2	
	s	s	s	-	-	-	4	
	s	s	s	-	s	-	1	(#46B)
	s	s	s	-	-	s	3	(#40)
	s	s	s	-	s	s	2	(#33)
	s	s	s	s	-	-	1	(#565)
	s	s	s	s	s	-	2	
	s	s	s	s	-	s	4	
	s	s	s	s	s	s	4	
								26
							total:	33

s indicates sensitivity
- indicates resistance

A - this group shows a change from the wild-type at 42C only
B - this group shows a change from the wild-type at both temperatures

These three antibiotics all affect cell wall synthesis in some way (Reynolds 1966). They are also three of the largest in molecular weight of the antibiotics tested. These results apparently support the idea of a defective cell envelope.

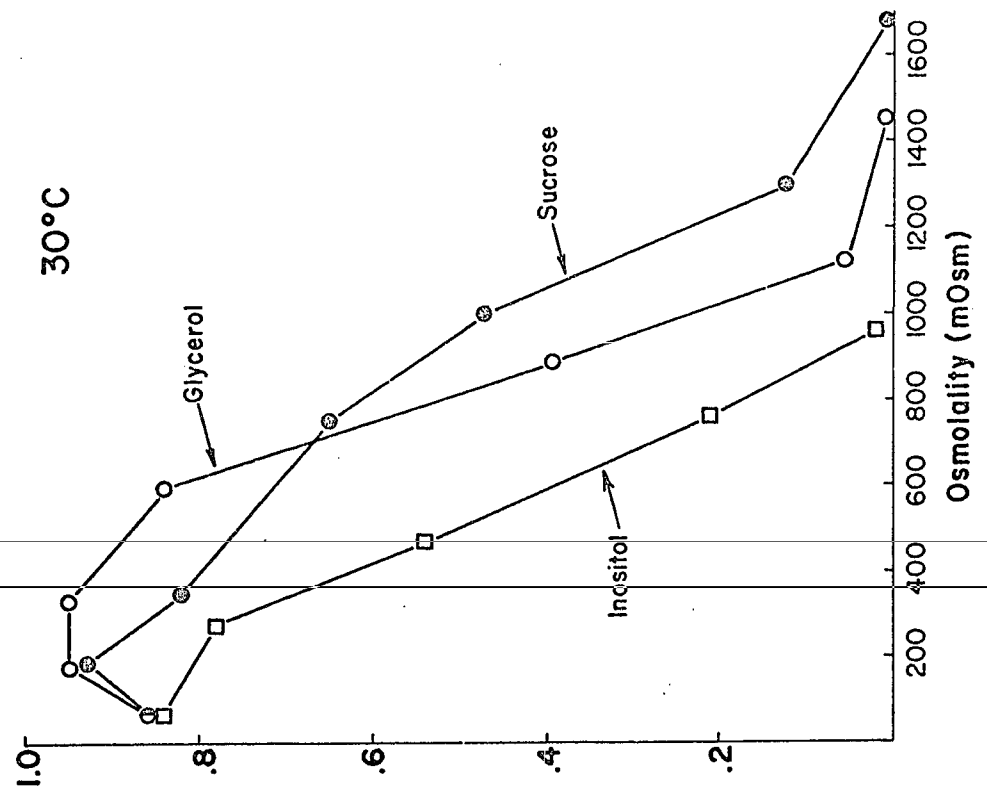
The majority of the mutants showed similar behaviour; thirty-three were "osmotically suppressible" at 42C, and all but one of these showed differences in antibiotic sensitivity from the wild-type. Several were selected for detailed characterization as described below. The greatest amount of work was done on mutant 33, which was in Group V on complex media and showed sensitivity differences at both temperatures.

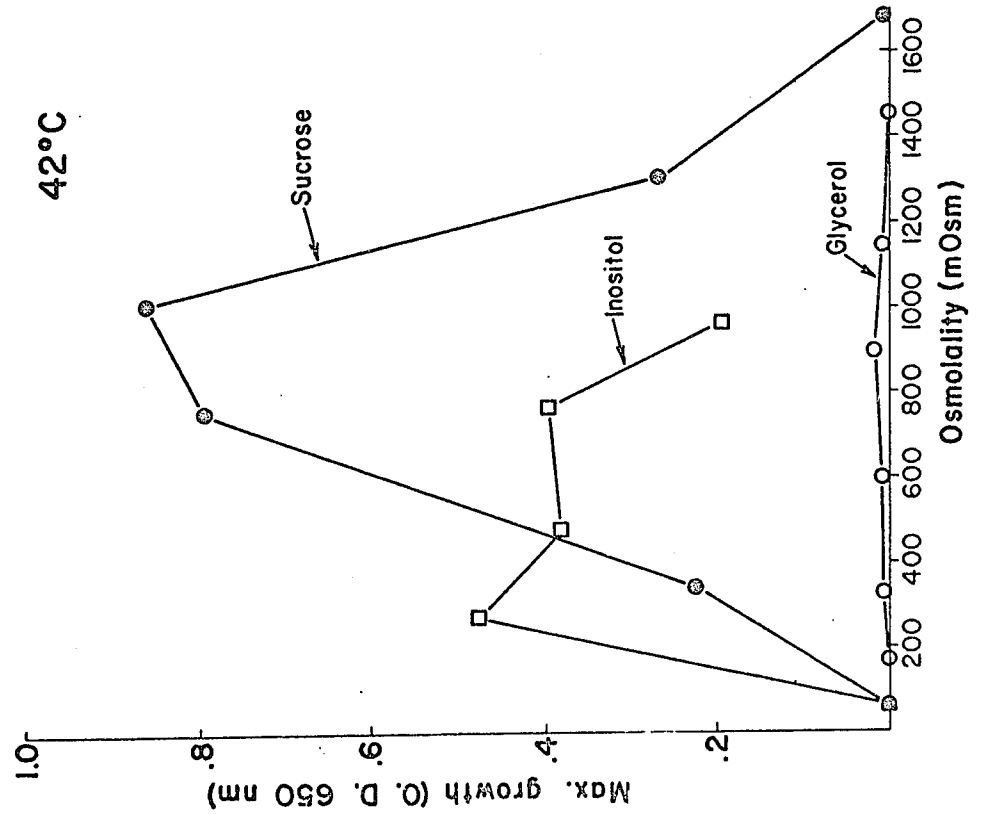
OSMOLALITY PROFILES

When the mutants were classified, NaCl was added to increase osmolality. What other compounds would permit growth at the non-permissive temperature? To test this quantitatively, tryptone broth was chosen as the basic medium. Two classes of compounds were added to it: (i) one of three salts - KCl, NaCl or $MgCl_2$; or (ii) one of three carbohydrates - glycerol, inositol or sucrose. Standard curves of concentration versus osmolality were linear in all six cases.

Figure 3 shows the osmolality profiles obtained at both 30C and 42C for mutant 33 with these three carbohydrates. Glycerol did

Figure 3 - Osmolality profiles of mutant 33 with glycerol, inositol and sucrose as osmotic agents. The graphs show maximum growth at different osmolalities at both 30C and 42C.


30°C
Glycerol
Sucrose
Inositol
1.0
.8
.6
.4
.2
200
400
600
800
1000
1200
1400
1600
Osmolality (mOsm)


42°C
Sucrose
Inositol
Glycerol
Max. growth (O. D. 650 nm)
1.0
.8
.6
.4
.2
200
400
600
800
1000
1200
1400
1600
Osmolality (mOsm)

not permit growth at the non-permissive temperature regardless of concentration. Since it is a small molecule that freely penetrates across the cell membrane, it may not have been exerting any osmotic effect. When inositol was present, growth was relatively poor at both temperatures except for very low concentrations at 30C. Although inositol is metabolized by E. coli, it apparently had a toxic effect at concentrations above 0.2M (268 mOsm). A solubility problem was also encountered here; a 1M solution (1060 mOsm) is supersaturated at room temperature. Sucrose was the least effective of the three. It gave nonreproducible results and tended to cause clumping of the cells, particularly at 42C. As with inositol there was a solubility problem, since very high concentrations of sucrose were required to produce this range of osmolality.

Figure 4 shows the osmolality profiles obtained for mutant 33 with the three salts as osmotic agents. All three allowed growth at the non-permissive temperature. However $MgCl_2$ allowed growth within a much narrower range of osmolality than either KCl or NaCl. Since this occurred at 30C as well, the three salts appear to be exerting a similar effect. KCl and NaCl gave almost identical profiles, except that KCl allowed growth at 42C at a lower osmolality than did NaCl. In the case of another mutant, #565, this difference did not occur (Figure 5).

The previous profiles compared the responses of one mutant to different osmotic agents. The following profiles were done to compare the responses of different mutants to the same osmotic agent, in this case NaCl. All the mutants studied gave similar growth curves;

Figure 4 - Osmolality profiles of mutant 33 with KCl, NaCl and $MgCl_2$ as osmotic agents. The graphs show maximum growth at different osmolalities at both 30C and 42C.

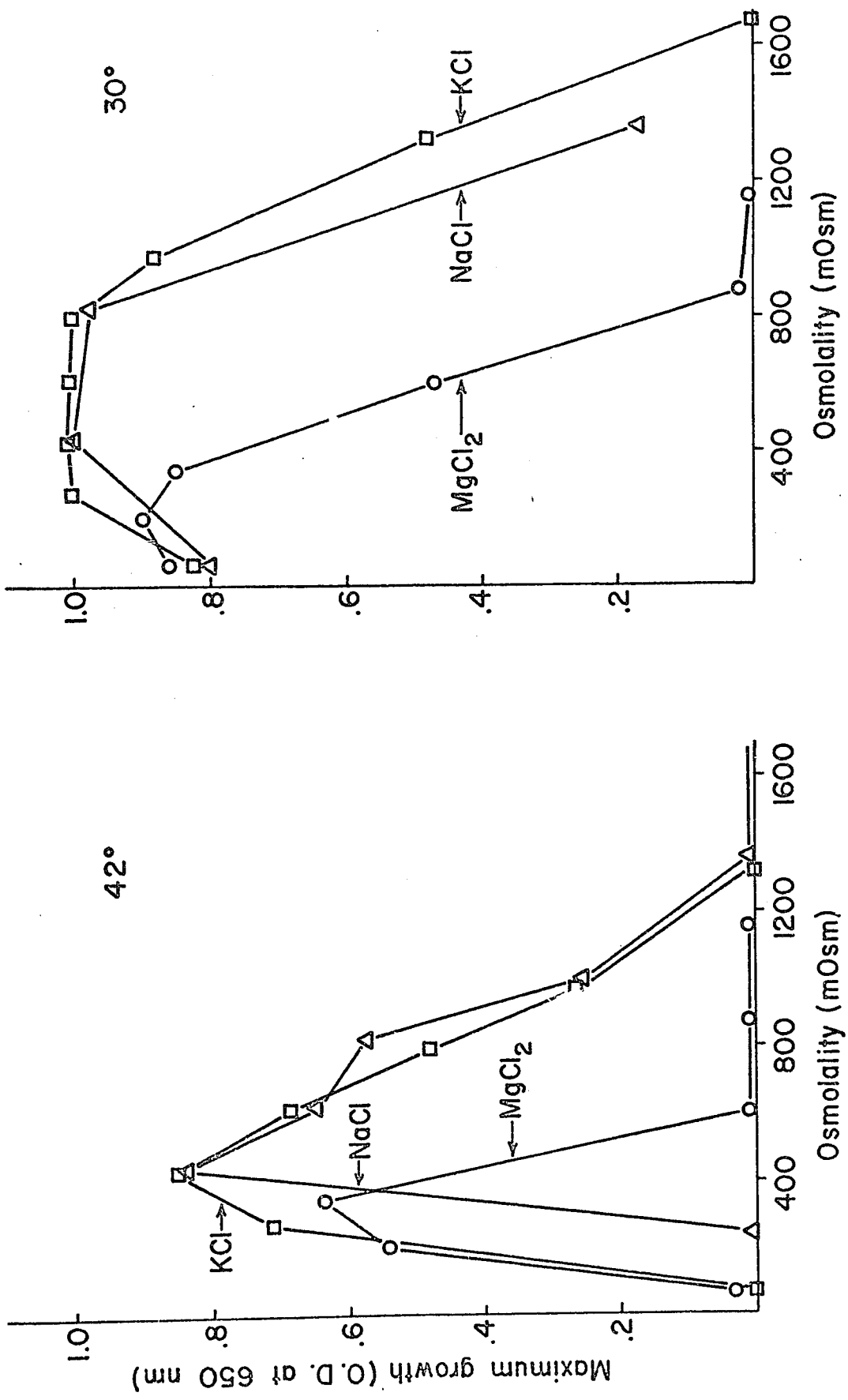
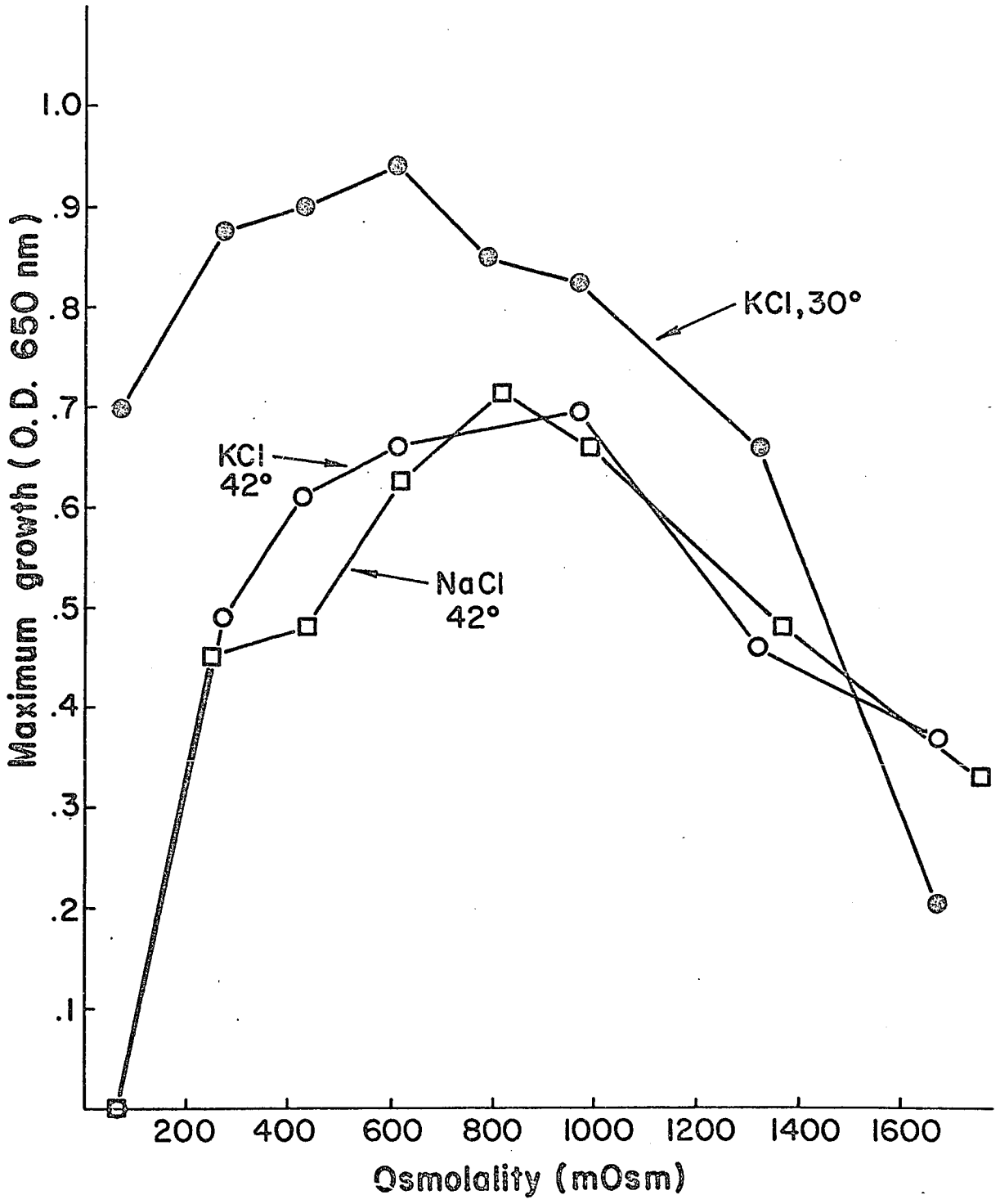

Maximum growth (O. D. at 650 nm)
1.0
.8
.6
.4
.2
42°
KCl
NaCl
MgCl2
400
800
1200
1600
Osmolality (mOsm)
30°
KCl
NaCl
MgCl2
400
800
1200
1600
Osmolality (mOsm)

Figure 5 - Comparison of osmolality profiles obtained for mutant 565 at 42C with NaCl and KCl as osmotic agents. KCl was used in the profile obtained at 30C.


Maximum growth (O.D. 650 nm)
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
200
400
600
800
1000
1200
1400
1600
Osmolality (mOsm)
KCl, 30°
KCl
42°
NaCl
42°

those obtained for mutant 46B were typical (Figure 6). Figure 7 shows the profiles obtained at 42C for four different mutants (including 33) and that of the parent; maximum growth was plotted here for a direct comparison. In Figure 8, maximum growth of the mutants was plotted as a percentage of that of the wild-type (AO14) at the same osmolality. Three of the mutants showed no growth at either extreme of osmolality while mutant 565 parallelled the response of the parent at higher osmolalities only (Figure 8). Although all mutants had their own distinct profiles, all exhibited the same ability to be "osmotically suppressed" within a certain range of osmolality.

SHIFT EXPERIMENTS

To test the possiblity of defective cell envelopes, several temperature shift experiments were done to see if lysis would occur. Cultures of mutants 40 and 46B were shifted to 42C after growth to mid-exponential phase at 30C. After thirty hours at 42C there was no decrease in O.D. (Figure 9). Microscopic observation showed only a decrease in motility; this was a subjective evaluation. Three identical cultures of mutant 33 were also shifted: one in early log phase, a second in mid-log phase and a third in stationary phase (Figure 10). Unexpectedly, there was an initial increase in O.D. followed by a levelling off in cultures shifted before reaching stationary phase. This may have been partly due to an elongation of the cells after shifting. The viable counts did not change significantly in these

Figure 6 - Growth curves for mutant 46B at different osmolalities at 42C. The highest osmolality used (1738 mOsm) corresponds to a concentration of 0.9M NaCl in tryptone broth.

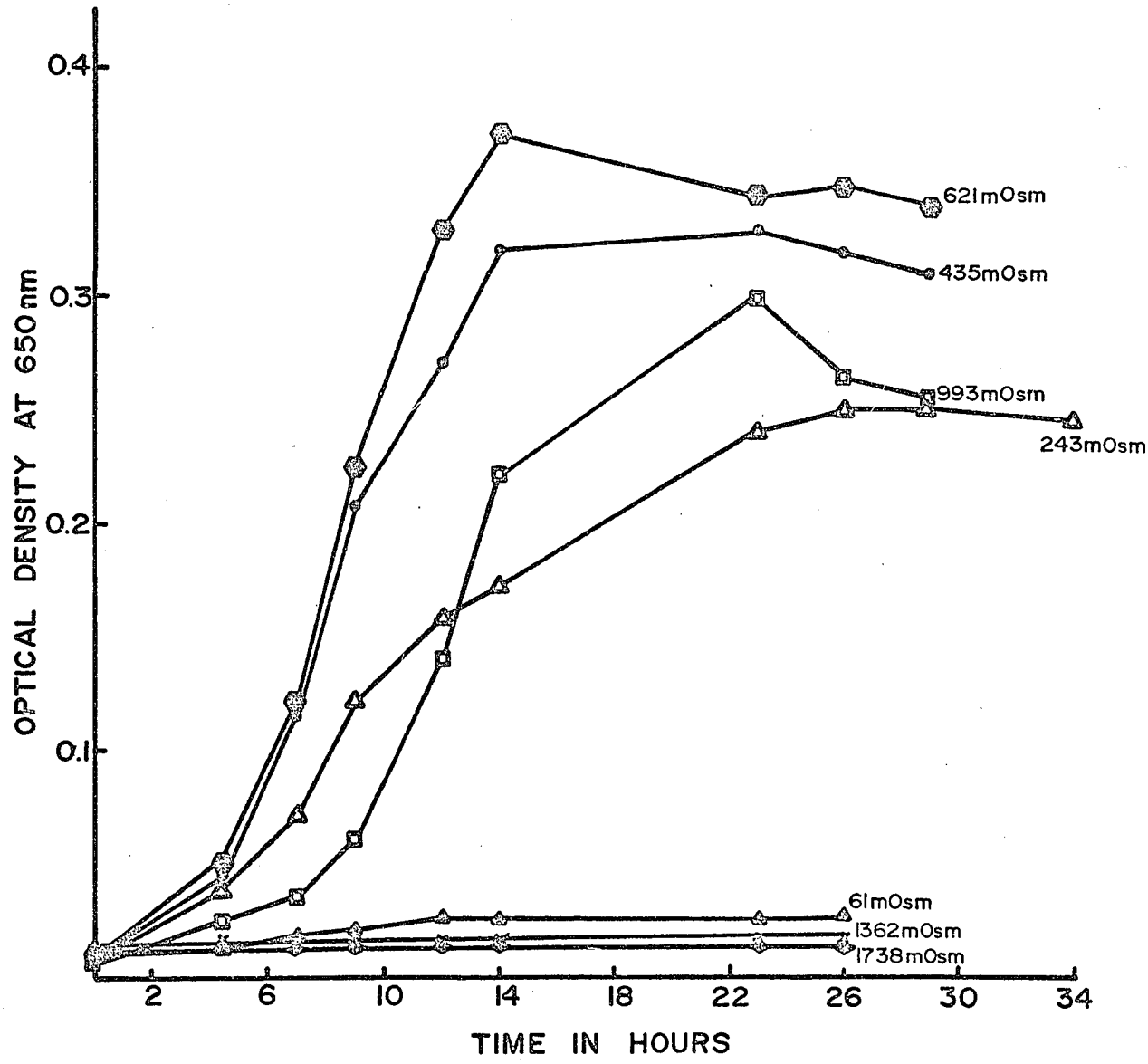

OPTICAL DENSITY AT 650 nm
0.4
0.3
0.2
0.1
621mOsm
435mOsm
993mOsm
243mOsm
61mOsm
362mOsm
1738mOsm
2
6
10
14
18
22
26
30
34
TIME IN HOURS

Figure 7 - Osmolality profiles obtained for mutants 33, 565, 46B and 40 and for the parent (AOl4) at 42C in tryptone broth with NaCl.

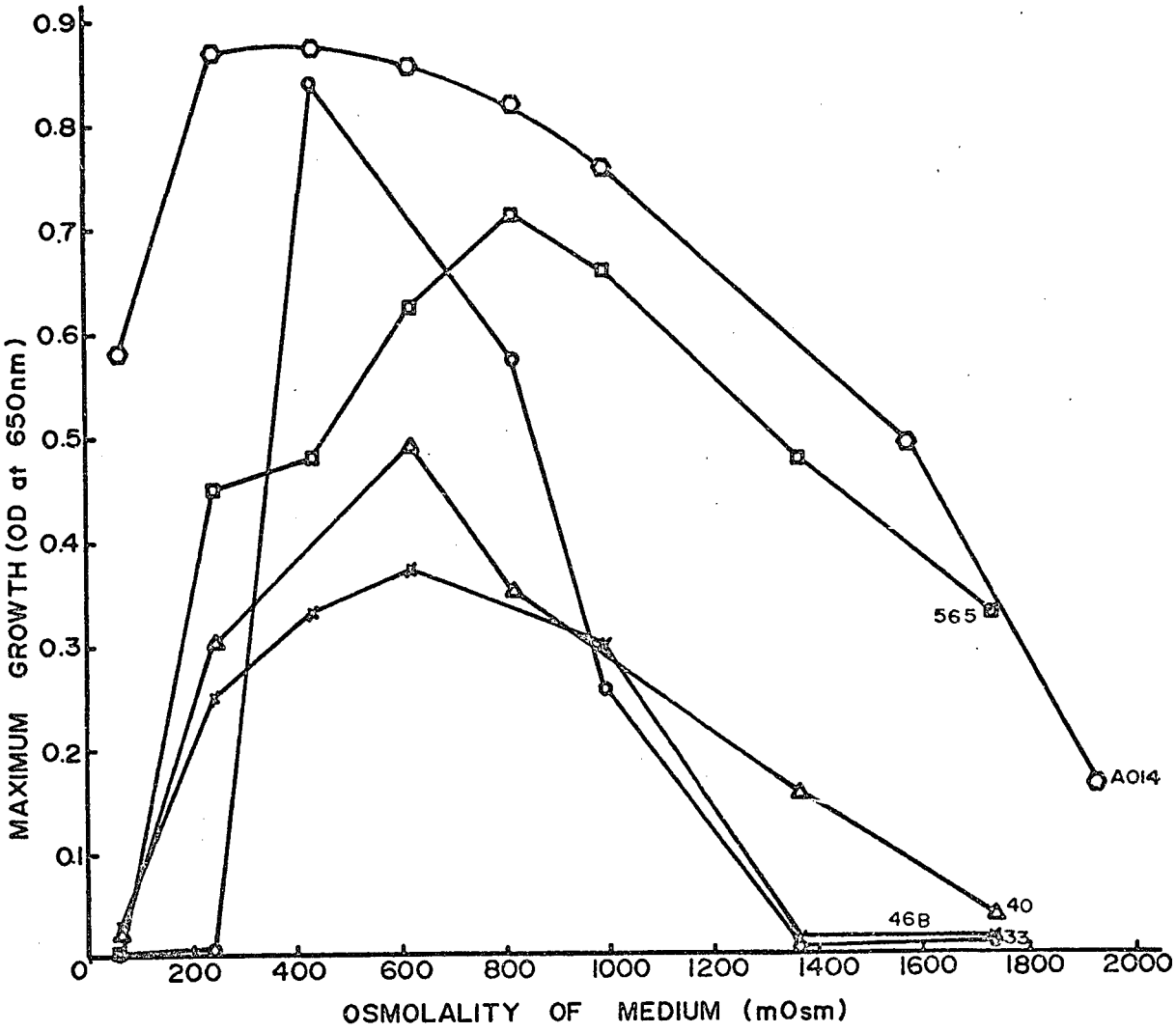

MAXIMUM GROWTH (OD at 650nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
200
400
600
800
1000
1200
1400
1600
1800
2000
OSMOLALITY OF MEDIUM (mOsm)
565
A014
40
468
33

Figure 8 - Osmolality profiles for mutants 33, 565, 46B and 40 at 42C in tryptone broth with NaCl. Maximum growth is plotted as a percentage of the wild-type (AO14) at the same osmolality.

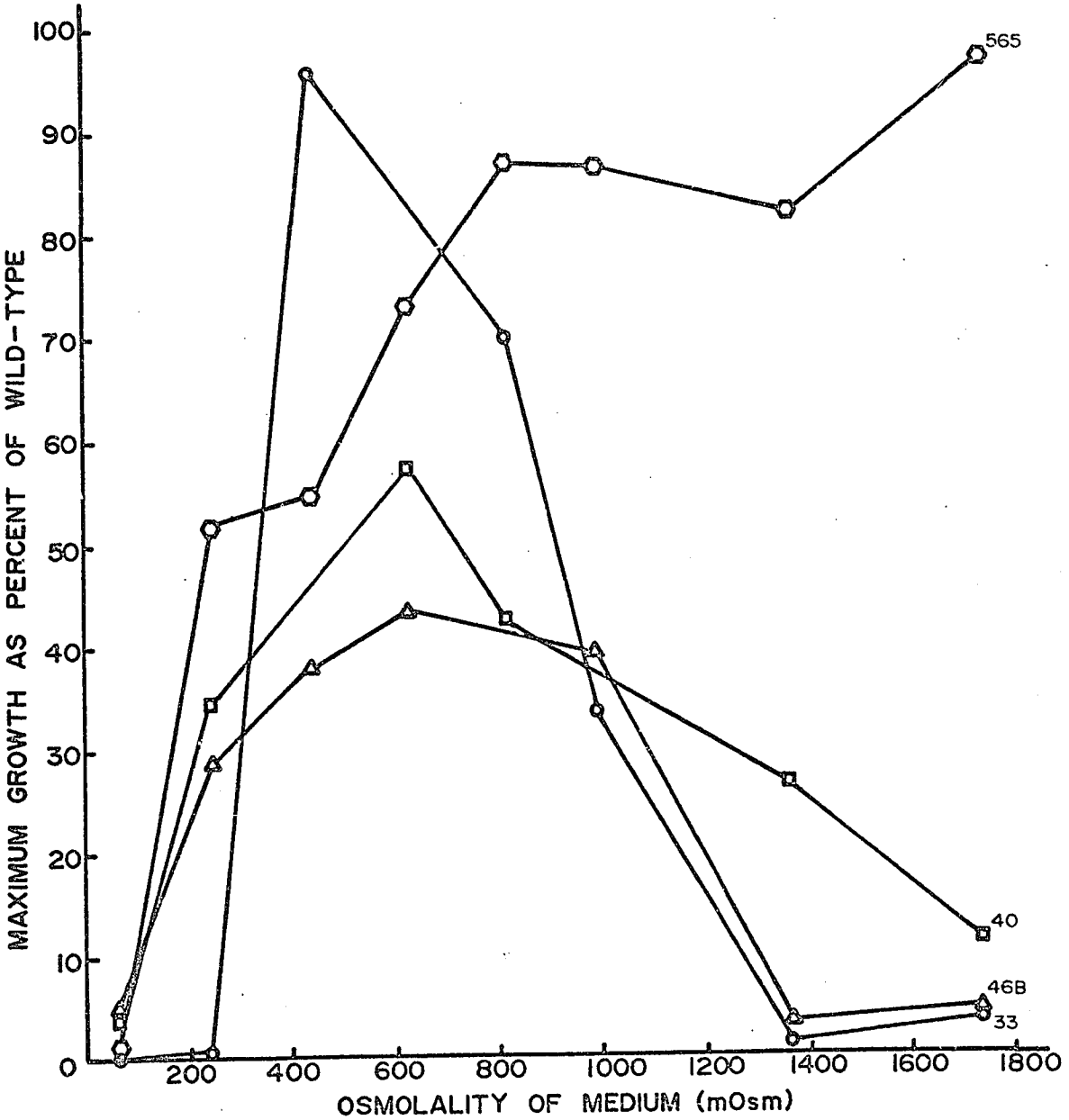

MAXIMUM GROWTH AS PERCENT OF WILD-TYPE
OSMOLALITY OF MEDIUM (mOsm)
0
10
20
30
40
50
60
70
80
90
100
200
400
600
800
1000
1200
1400
1600
1800
565
40
46B
33

Figure 9 - Changes in $O.D._{650}$ for mutants 40 and 46B after shifting to 42C. The mutants were grown to mid-log phase at 30C in tryptone broth. The microscopic observations on motility were subjective.

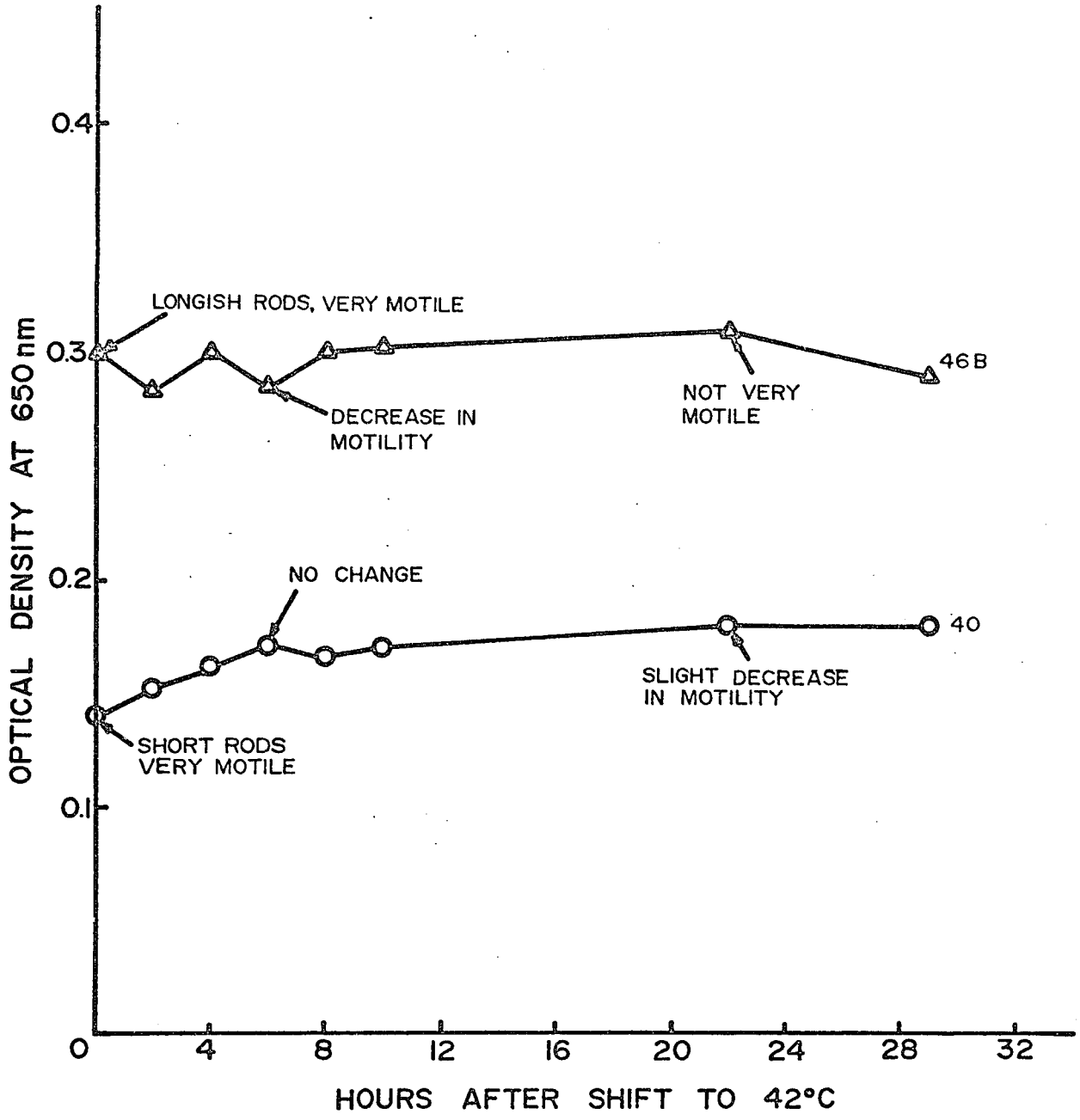
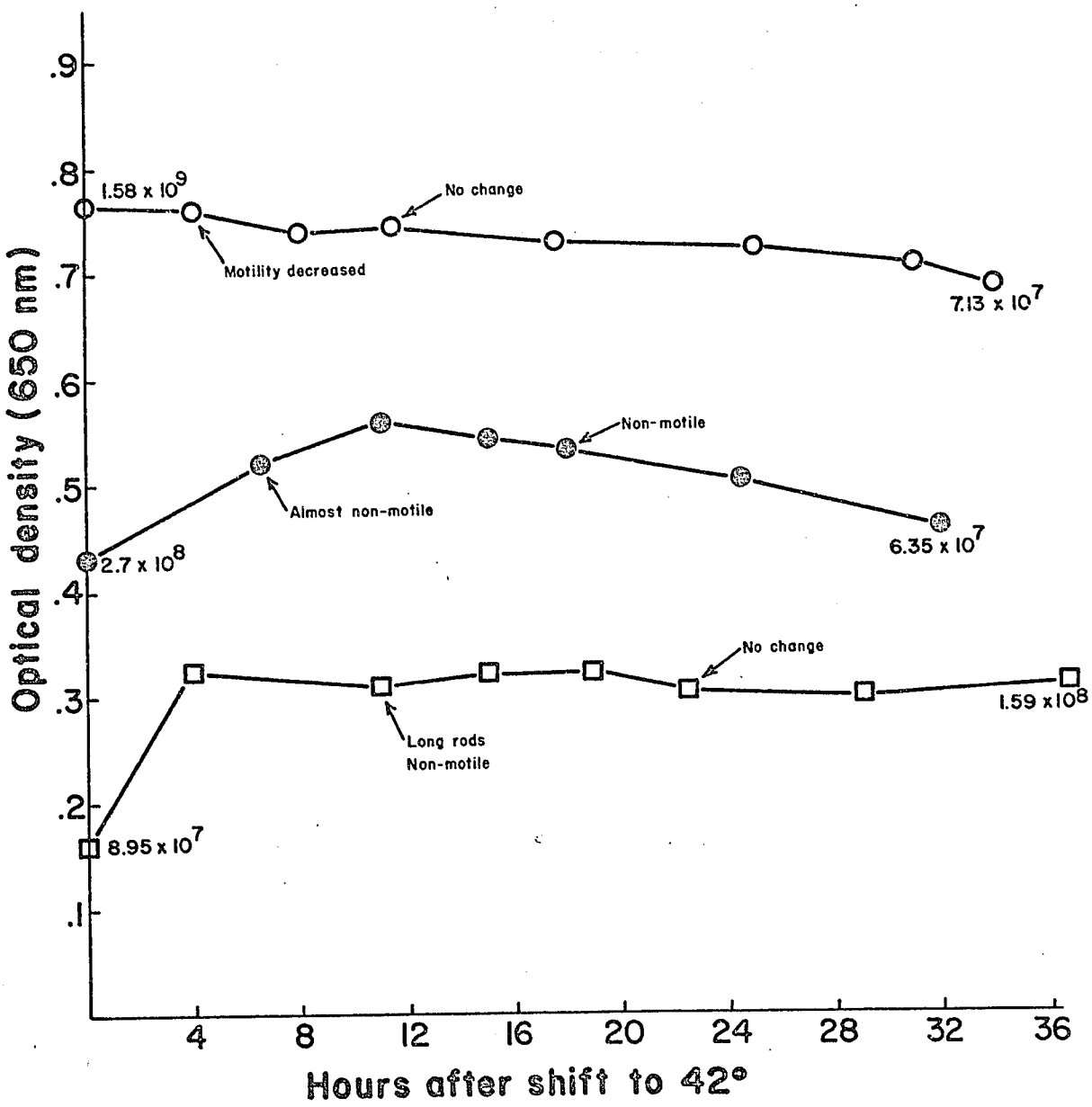

0.4
0.3
0.2
0.1
0
OPTICAL DENSITY AT 650 nm
LONGISH RODS, VERY MOTILE
DECREASE IN MOTILITY
NOT VERY MOTILE
46B
NO CHANGE
SLIGHT DECREASE IN MOTILITY
40
SHORT RODS VERY MOTILE
4
8
12
16
20
24
28
32
HOURS AFTER SHIFT TO 42°C

Figure 10 - Changes in $O.D._{650}$ for mutant 33 after shifting to 42C. Observations on motility were subjective.


Optical density (650 nm)
.9
.8
.7
.6
.5
.4
.3
.2
.1
1.58 x 10^9
No change
Motility decreased
7.13 x 10^7
Non-motile
Almost non-motile
2.7 x 10^8
6.35 x 10^7
No change
1.59 x10^8
Long rods
Non-motile
8.95 x 10^7
4
8
12
16
20
24
28
32
36
Hours after shift to 42°

cultures even after thirty-six hours at 42C.

In a second experiment with mutant 33, one culture was shifted in early log phase, another in mid-log phase, then both were shifted back to 30C after different lengths of time at the non-permissive temperature. Figure 11 shows that the critical parameter was the length of time at 42C. The ability of the cells to multiply at 30C decreased as the length of time at 42C increased. After 12 hours at 42C, their ability to recover was considerably less. The initial increase in O.D. at 42C corresponded to a negligible increase in cell numbers (Table 3), probably being due to elongation of the cells. Since the O.D. remained approximately constant for 48 hours at 42C, the cell envelope appeared to be quite stable at the non-permissive temperature.

In another shift experiment, NaCl was added to cultures of mutants 33 and 565 while they remained at 42C. Figure 12 shows the response of mutant 565; that of mutant 33 was similar. The O.D. dropped after 3 hours and the viable counts decreased considerably. NaCl thus appeared to precipitate the disintegration of the cells at the non-permissive temperature.

Figure 11 - Shift experiment with mutant 33. Two cultures were shifted to 42C, then shifted back to 30C (dotted lines) after 6 hours, 12 hours and 21 hours. In Figure 11A, the culture was shifted to 42C in late log phase. In Figure 11B, the culture was shifted to 42C in early log phase.

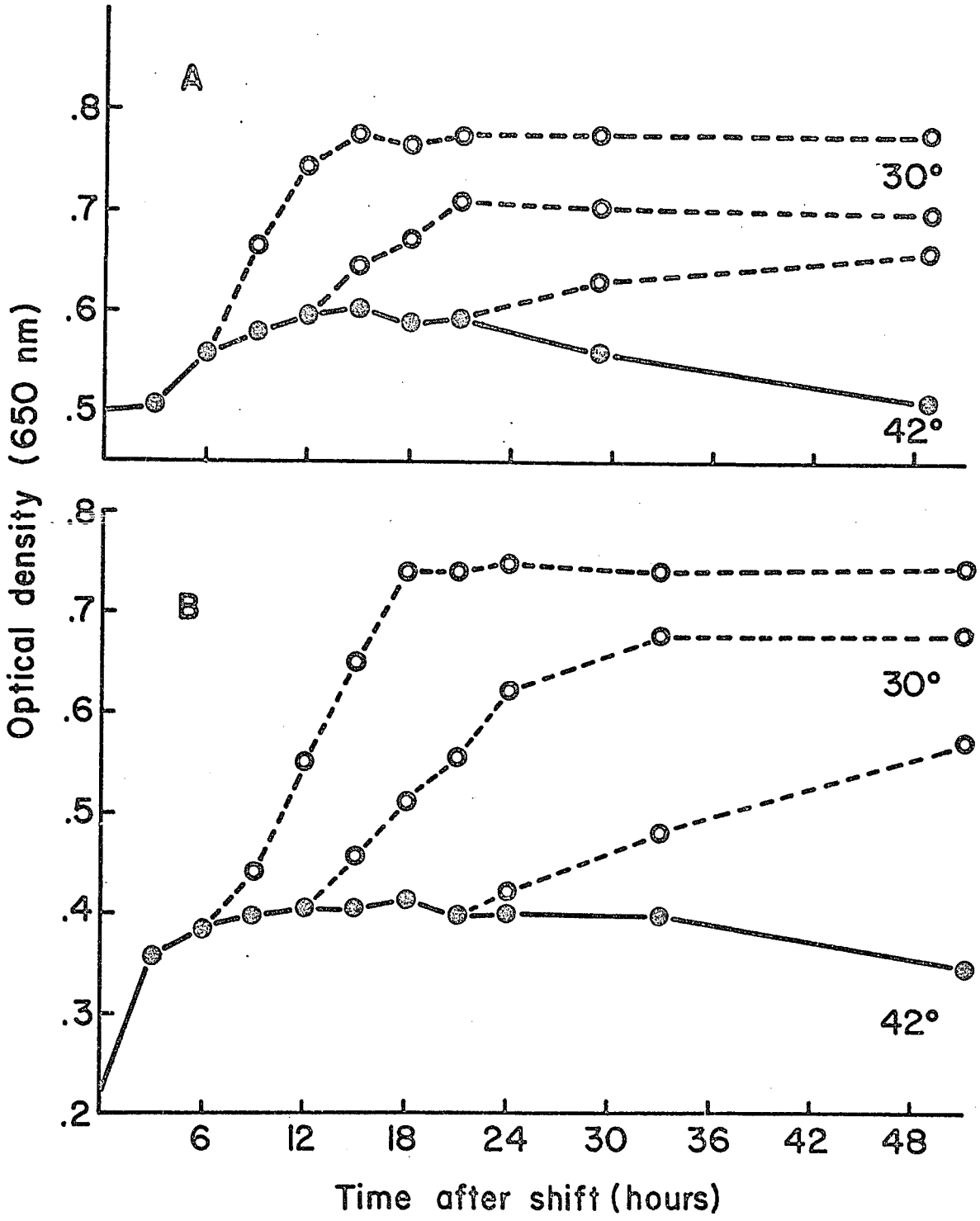

A
B
30°
42°
30°
42°
Optical density (650 nm)
.8
.7
.6
.5
.4
.3
.2
6
12
18
24
30
36
42
48
Time after shift (hours)

TABLE 3

Viable counts for shift experiment with mutant #33

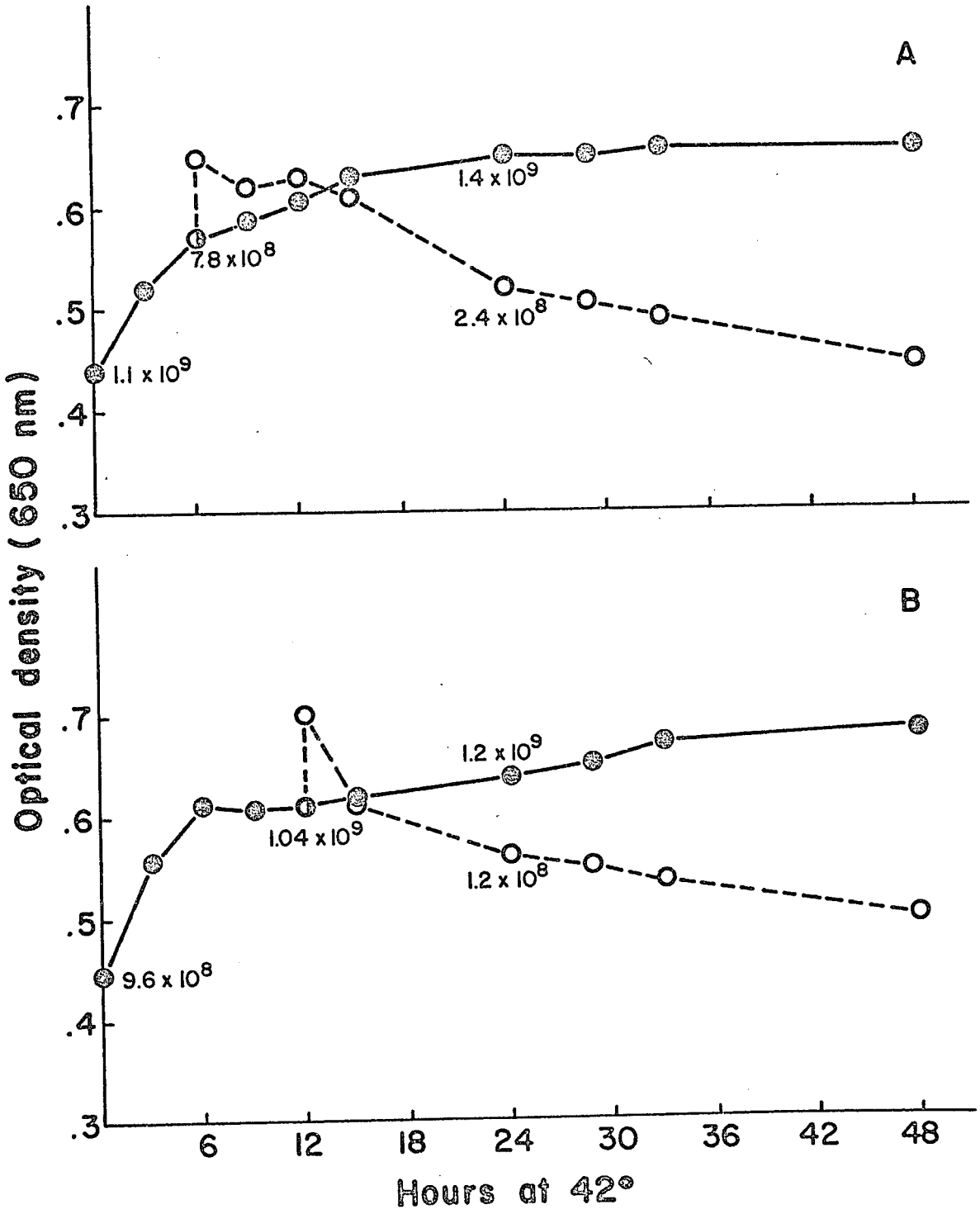
(see Figure 11)

	hours at 42C	viable count (no. cells/ml)	culture shifted back to 30C after 6 hours at 42C	culture shifted back to 30C after 12 hours at 42C
A	0	1.0×10^9		
	6	1.0×10^9		
	12	9.5×10^8	1.5×10^9	
	21	5.0×10^8	1.8×10^9	1.0×10^9
B	0	3.0×10^8		
	6	3.7×10^8		
	12	3.0×10^8	7.0×10^8	
	21	2.0×10^8	1.7×10^9	4.8×10^8

A - culture shifted to 42C in late log phase; corresponds to Figure 11A

B - culture shifted to 42C in early log phase; corresponds to Figure 11B

Figure 12 - Effect of adding NaCl to cultures of mutant 565 after shift to 42C. The dotted lines show changes in $O.D._{650}$ after the addition of NaCl. The numbers represent viable counts (no. cells per ml). In Figure 12A, NaCl was added after 6 hours at 42C. In Figure 12B, NaCl was added after 12 hours at 42C.


A
B
Optical density (650 nm)
.7
.6
.5
.4
.3
1.1 x 10^9
7.8 x 10^8
1.4 x 10^9
2.4 x 10^8
9.6 x 10^8
1.04 x 10^9
1.2 x 10^9
1.2 x 10^8
6
12
18
24
30
36
42
48
Hours at 42°

DISCUSSION

The results presented here show that, in some cases, temperature sensitivity can be overcome by increasing the osmotic concentration of the growth medium. In a group of 68 ts mutants selected on complete medium, slightly more than 50% were able to grow at the non-permissive temperature when the osmolality of the medium was increased. Three sets of experiments were done: first, the mutants were classified according to both osmotic requirements and differential sensitivity to antibiotics; second, the effectiveness of six different osmotic agents in reversing temperature sensitivity was examined; third, the hypothesis that the cell envelopes were defective was tested. All of the mutants were classified in the first set of experiments, after which four were studied in greater detail.

The mutants were classified in seven groups on the basis of their ability to grow on various complex media at 42C (Table 1). These media are all nutritionally complete and differ only in their osmotic concentration. Therefore, these seven groups appear to represent the

minimum osmolality requirements of the mutants for growth at the non-permissive temperature. The six Group I mutants exhibited a higher osmotic requirement than the other 35 mutants since four of them were able to grow on supplemented minimal medium at 42C, a medium of higher osmolality than those listed in the table. The Group VII mutants did not grow on penassay at 42C, yet did grow on media with lower osmolality. Perhaps some of these mutants have a very limited osmotic requirement for growth at 42C, or, as previously suggested, something in that medium inhibits growth at the non-permissive temperature. The identity of such a compound is impossible to guess.

Several mutants did not grow at 30C on minimal medium. Increasing the osmolality by the addition of 0.5% NaCl did not overcome either the nutritional requirement or the temperature sensitivity. In these cases the nutritional requirement was ignored, since it could not have been the cause of the temperature sensitivity.

Many of the mutants showed abnormal morphology when growing at 42C with added NaCl. Cells with faulty septum formation or alterations in cell division tend to produce long "snakes" and have altered permeability (Kohiyama et al. 1966). As a result, they show differential sensitivity to various antibiotics (Normark 1969; Normark et al. 1969). As a preliminary test of this possibility and a further classification, the mutants were tested for sensitivity to ten different antibiotics.

All the mutants and the parent were sensitive to ampicillin,

chloramphenicol, nalidixic acid and kanamycin. These four antibiotics are the smallest in size, ranging in molecular weight from 232 (nalidixic acid) to 484 (kanamycin). Chloramphenicol inhibits protein synthesis, nalidixic acid inhibits DNA synthesis, kanamycin inhibits RNA synthesis and ampicillin affects cell wall synthesis. Presumably these four could easily penetrate the cells and disrupt cellular processes. The resistance of all mutants and the parent to valinomycin, lincomycin and streptomycin was not surprising. The parent carries a gene for streptomycin resistance which was obviously not affected by mutagenesis. Lincomycin affects protein synthesis (like chloramphenicol) but the concentration used here (2 μg) was probably too low to be effective. Valinomycin was dissolved in absolute alcohol and placed on a sensi-disc; it appears that only the alcohol diffused into the medium and not the antibiotic.

The mutants exhibited different responses than the parent to only three antibiotics - vancomycin, bacitracin and novobiocin (Table 2). These are three of the largest of the antibiotics tested (MW 3300, 1411 and 613 respectively) and they all affect cell wall synthesis in some way (Reynolds 1966). Vancomycin is thought to affect one of the processes involved in the polymerization of muramyl-peptides. Bacitracin causes a loss of membrane integrity as well as inhibiting cell wall synthesis. Novobiocin, on the other hand, inhibits protein and nucleic acid synthesis first but shows a lack of specificity of action. Apparently ion and membrane integrity are important and it does affect cell wall synthesis, although not primarily. Therefore a change in sensitivity to one or more of these antibiotics may reflect increased

permeability due to an alteration in the cell envelope.

The mutants were classified in two major groups on the basis of their response to these three antibiotics: (i) Group A became sensitive to one or more of these three only at the non-permissive temperature; (ii) Group B, the larger group, became sensitive to one or more of the three at both temperatures. Assuming that this is a valid test for altered cell envelopes, all but one of the mutants appear to have some kind of envelope defect. The Group A mutants may possess either a thermolabile structural protein or a thermolabile enzyme involved in the synthesis of an envelope component. The Group B mutants, however, appear to be structurally altered at both temperatures. A medium of higher osmolality could be stabilizing either defect, thus allowing growth at 42C.

Preliminary tests had indicated that the reversal of the temperature sensitivity by the presence of NaCl was an osmotic rather than ionic effect. As further proof, three carbohydrates and three salts were tested as osmotic agents with mutant 33, one of the more stable mutants.

The three carbohydrates chosen proved only partially effective. Inositol, although metabolized by E. coli, appeared to have a toxic effect on the cells at concentrations above 0.2M at both temperatures. As previously mentioned, there was a solubility problem with this compound since the concentrations required to obtain this range of osmolality caused the inositol to crystallize out of solution

at 30C. Glycerol, on the other hand, did not allow any growth at 42C. Since it freely penetrates across the cell membrane (Hayashi and Lin 1965), it would be ineffective as an osmotic agent. Sucrose did relieve the temperature sensitivity but the results were not reproducible. The clumping observed could have been the result of plasmolysis due to the high concentrations required (up to 50%). Since sucrose was an effective osmotic agent when a small amount was present in solid medium, the reversal of temperature sensitivity was still considered to be an osmotic effect.

The three salts - KCl, NaCl and $MgCl_2$ - all proved effective in relieving temperature sensitivity. However, $MgCl_2$ allowed growth within a much narrower range of osmolality than the other two salts at both temperatures, inhibiting growth at concentrations above 0.25M ($\sim$ 700 mOsm) at 30C and above 0.2M ($\sim$ 500 mOsm) at 42C. At 42C, KCl allowed growth at a lower concentration than did NaCl. However, this difference did not occur with a second mutant (565) and may simply be an unusual feature of mutant 33.

Two other mutants were also tested with NaCl to obtain osmolality profiles at 42C. Of the four mutants tested, only 565 paralleled the response of the parent at higher osmolalities. Growth of the other three mutants at 42C was inhibited at both high and low osmolalities. Each profile was slightly different, with mutant 33 showing the highest minimum requirement. This was the only case where the minimum osmolality for growth at 42C was higher than that predicted by the original classification on solid medium. These profiles show conclusively that these mutants can be "osmotically suppressed".

The evidence to this point suggested a structural defect in the cell envelopes of these mutants. Therefore, if cultures grown at 30C in a medium of low osmolality were shifted to 42C, lysis might be expected to occur. When cultures of four different mutants (all in Group B for antibiotic sensitivity) were shifted this way, the O.D. remained constant for as long as 36 hours at 42C with only a slight decrease in viability. The cells also retained the ability to grow at 30C after as long as 21 hours at 42C (Figure 11 and Table 3). Surprisingly, the O.D. initially increased when the cultures were shifted to 42C. However, this was due to an elongation of the cells rather than an increase in cell numbers (see Figure 10). It appears, therefore, that macromolecular synthesis can continue at 42C while some other aspect of cell division (septum formation?) cannot. Thus the cell envelope appeared to be structurally stable at the non-permissive temperature. On the other hand, addition of NaCl to cultures previously shifted to 42C caused a decrease in viability almost immediately. The initial increase in O.D. was probably caused by a shrinking of the cells (see Figure 12; Bernheim 1963). This experiment suggested that the cell envelopes were somehow defective, since the cells could no longer tolerate a change in osmolality.

These results appear to contradict each other. According to the test for antibiotic sensitivity, the mutants are defective somewhere in the cell envelope. According to the shift experiments, the mutants have stable cell envelopes. When cultures are shifted to the

non-permissive temperature in a medium of low osmolality, they can elongate but not divide, yet they are able to divide when returned to the permissive temperature. There is insufficient evidence for a positive conclusion but there is a possible explanation.

Since cell division depends on DNA synthesis (Clark 1968), mutants ts for DNA synthesis often form long filaments at the non-permissive temperature (Kohiyama et al. 1966). However, such mutants often have membrane defects, since DNA synthesis is thought to be membrane-associated (Siccardi et al. 1971; Inouye and Guthrie 1969). The mutants reported here could be defective in membrane structure. The differential sensitivity to antibiotics could be a permeability effect, similar to the effects reported for the cet, tol and envA genes (Holland et al. 1970; Whitney 1971; Normark 1969). Another of the effects of a mutation in any of these genes is faulty cell division. Therefore it is possible that the mutants described here contain a thermolabile membrane component. This component could be structurally altered in such a way that it causes permeability changes at both temperatures as well as causing failure of division at the non-permissive temperature (assuming that this component is at or near the site of DNA replication on the cell membrane; Jacob et al. 1966). A higher osmotic concentration in the medium might relieve this type of defect by stabilizing the configuration of the altered component. As long as the cell envelope maintained its integrity, no lysis would be observed after a shift to the non-permissive temperature in a medium of low osmolality even though the membrane was altered. Similarly,

adding NaCl after the shift would cause a loss of water from the cells, followed by a decrease in O.D. and cell death (Bernheim 1963), since the membrane would no longer be able to function effectively as a permeability barrier.

There is another related possibility. Several workers have postulated the existence of a protein called a "division activator" that is necessary for cell division to occur (Walker and Smith 1970; Smith and Pardee 1970). Such a protein would be lacking in mutants unable to form septa (Walker and Smith 1970). Normark (1969) reported mutants of E. coli defective in the envA gene; these could not form septa, thus forming long chains, and had decreased resistance to several antibiotics, one of which was novobiocin. If a "division activator" does exist, then the mutants described by Normark would also be lacking in this protein. The mutants described here could be temperature sensitive for septum formation with a thermolabile "division activator". Since the septum forms from the cell membrane, the membrane could be altered at both temperatures, causing permeability changes. Presumably such a defect could be stabilized by a higher osmotic environment, allowing macromolecular synthesis and growth to continue at the non-permissive temperature.

It is unlikely that these mutants are temperature sensitive for protein synthesis. Some such mutants show abnormal morphology at the non-permissive temperature while others die after a shift to the non-permissive temperature (Russell and Pittard 1971b). If an enzyme

required for biosynthesis was thermolabile, then the cells should appear abnormal only at the non-permissive temperature. The mutants studied in the shift experiments were all, unfortunately, in Group B for antibiotic sensitivity, i.e. they showed differential sensitivity at both temperatures. From these experiments it is impossible to rule out the possibility of ts protein synthesis, especially for the Group A mutants, but the evidence is weak.

Similarly, it is also unlikely that these mutants are ts for DNA synthesis itself. Such mutants form long filaments at the non-permissive temperature and do recover if shifted back to the permissive temperature, but only if left at the non-permissive temperature for short periods of time (Worcel 1970). Incorporation studies would have to be done, however, to prove that DNA synthesis does not stop at 42C in these mutants.

At the present time, the only reasonable conclusion that can be reached is that, in these mutants, the cell membrane is defective. Perhaps a structural component of the membrane is thermolabile and is stabilized by the higher osmotic environment. Perhaps the membrane is structurally altered at both temperatures and a "division activator" is thermolabile, the latter being stabilized by the higher osmolality. Similarly, septum formation may be temperature sensitive as a result of a membrane defect. Although it appears that macromolecular synthesis can continue at the non-permissive temperature, incorporation studies must be done to see whether this is both DNA and protein synthesis or

only protein synthesis. It is also possible that the temperature sensitivity is caused by a suppressor gene such as that reported by Apirion (1966). This possibility was not discussed since there is absolutely no proof of its existence in this case. To prove that a membrane defect really is responsible for the temperature sensitivity, further experiments must be done, specifically phage adsorption and permeability studies.

Although no strong conclusion can be reached, it is interesting that a large percentage of randomly-selected ts mutants can be "osmotically suppressed", regardless of the exact defect involved. Indeed, these experiments suggest that osmotic reversal of temperature sensitivity could be a more general phenomenon in microorganisms than previously thought. Perhaps further testing of diverse ts mutants in media of varying osmolality is warranted.

REFERENCES

Apirion, D. 1966. Altered ribosomes in a suppressor strain of Escherichia coli. J. Mol. Biol. 16: 285-301.

Atherly, A.G. and Suchanek, M.C. 1971. Characterization of mutants of Escherichia coli temperature sensitive for ribonucleic acid regulation: an unusual phenotype associated with a phenylalanyl transfer ribonucleic acid synthetase mutant. J. Bacteriol. 108: 627-638.

Bernheim, F. 1963. Factors which affect the size of the organisms and the optical density of suspensions of Pseudomonas aeruginosa and Escherichia coli. J. Gen. Microbiol. 30: 53-58.

Bhattacharyya, P. 1970. Active transport of manganese in isolated membranes of Escherichia coli. J. Bacteriol. 104: 1307-1311.

Böck, A. 1968. Relation between subunit structure and temperature sensitivity of mutant phenylalanyl RNA synthetases of Escherichia coli. Eur. J. Biochem. 4: 395-400.

Boyd, D., Nixon, R., Gillespie, S. and Gillespie, D. 1968. Screening of Escherichia coli temperature sensitive mutants by pre-treatment with glucose starvation. J. Bacteriol. 95: 1040-1050.

Brockman, R.W. and Heppel, L.A. 1968. On the localization of alkaline phosphatase and cyclic phosphodiesterase in Escherichia coli. Biochemistry 7: 2554-2562.

Buller, C.S. and Dobbs, K. 1971. T4-coliphage infection of Escherichia coli with defective cell envelopes. Biochem. Biophys. Res. Comm. 43: 658-665.

Carl, P.L. 1970. Escherichia coli mutants with temperature sensitive synthesis of DNA. Molec. Gen. Genetics 109: 107-122.

Clark, D.J. 1968. Regulation of deoxyribonucleic acid replication and cell division in Escherichia coli B/r. J. Bacteriol. 96: 1214-1224.

Cooper, S. and Helmstetter, C.E. 1968. Chromosome replication and the division cycle of Escherichia coli B/r. J. Mol. Biol. 31: 519-539.

Crandall, M. and Koch, A.L. 1971. Temperature sensitive mutants of Escherichia coli affecting β-galactoside transport. J. Bacteriol. 105: 609-619.

Delgano, L. and Sinsheimer, R.L. 1968. Process of infection with bacteriophage ϕX174: XXIV - New type of temperature sensitive mutant. J. Virology 2: 822-829.

deLucia, P. and Cairns, J. 1969. Isolation of an Escherichia coli strain with a mutation affecting DNA polymerase. Nature 224: 1164-1166.

dePetris, S. 1967. Ultrastructure of the cell wall of Escherichia coli and chemical nature of its constituent layers. J. Ultrastruct. Res. 19: 45-83.

Edgar, R.S. and Epstein, R.H. 1965. Conditional lethal mutations in bacteriophage T4. Genetics To-day 2: 1-16.

Eggertsson, G. 1968. Suppressors causing temperature sensitivity of growth in Escherichia coli. Genetics 60: 269-280.

Fangman, W.L. and Russel, M. 1971. X-Irradiation sensitivity in Escherichia coli defective in DNA replication. Molec. Gen. Genetics 110: 332-347.

Forsberg, C.W., Costerton, J.W. and MacLeod, R.A. 1970. Quantitation, chemical characteristics, and ultrastructure of the three outer cell wall layers of a gram-negative bacterium. J. Bacteriol. 104: 1354-1368.

Fox, C.F. and Kennedy, E.P. 1965. Specific labelling and partial purification of the M protein, a component of the β-galactoside transport system of Escherichia coli. Proc. Nat. Acad. Sci. (U.S.) 54: 891-899.

Gallucci, E., Pacchetti, G. and Zangrossi, S. 1970. Genetic studies on temperature sensitive nonsense suppression. Molec. Gen. Genetics 106: 362-370.

Good, C.M. and Pattee, P.A. 1970. Temperature sensitive osmotically fragile mutants of Staphylococcus aureus. J. Bacteriol. 104: 1401-1403.

Gorini, L. and Beckwith, J.R. 1966. Suppression. Annual Review of Microbiology 20: 401-422.

Hawthorne, D.C. and Friis, J. 1964. Osmotic-remedial mutants. A new classification for nutritional mutants in yeast. Genetics 50: 829-839.

Hayashi, S. and Lin, E.C.C. 1965. Capture of glycerol by cells of Escherichia coli. Biochim. Biophys. Acta 94: 479-487.

Hirota, Y., Jacob, F., Ryter, A., Buttin, G. and Nakai, T. 1968. On the process of cellular division in Escherichia coli I. Asymmetrical cell division and production of deoxyribonucleic acid-less bacteria. J. Mol. Biol. 35: 175-192.

Hirota, Y., Mordoh, J. and Jacob, F. 1970. On the process of cellular division in Escherichia coli III. Thermosensitive mutants of Escherichia coli altered in the process of DNA initiation. J. Mol. Biol. 53: 369-387.

Holland, I.B., Threlfall, E.J., Holland, E., Darby, V. and Samson, A. 1970. Mutants of Escherichia coli with altered surface properties which are refractory to colicin E2, sensitive to ultraviolet light and which can also show recombination deficiency, abortive growth of bacteriophage lambda and filament formation. J. Gen. Microbiol. 62: 371-382.

Horowitz, N.H. 1950. Biochemical genetics of Neurospora. Adv. Genetics 3: 33-71.

Horowitz, N.H. and Leupold, U. 1951. Some recent studies bearing on the one gene-one enzyme hypothesis. Cold Spring Harbour Symposium on Quantitative Biology 16: 65-74.

Inouye, M. and Guthrie, J.P. 1969. A mutation which changes a membrane protein of Escherichia coli. Proc. Nat. Acad. Sci. 64: 957-961.

Irr, J. and Englesberg, E. 1971. Control of expression of the L-arabinose operon in temperature sensitive mutants of gene araC in Escherichia coli B/r. J. Bacteriol. 105: 136-141.

Jacob, F., Ryter, A. and Cuzin, F. 1966. On the association between DNA and the membrane in bacteria. Proc. Roy. Soc. London B 164: 267-278.

Kanamitsu, O. and Ikeda, Y. 1969. Genetics of the temperature-dependent and -sensitive revertants obtained from threonine-requiring mutants of Bacillus subtilis. J. Gen. Appl. Microbiol. 15: 463-471.

Kanamitsu, O., Kuroda, Y., Saito, H. and Ikeda, Y. 1970. A mutant of Bacillus subtilis appearing to have a temperature sensitive defect in the ribosomes. J. Biochem. 67: 353-361.

Kang, S.-S. 1970. A mutant of Escherichia coli with temperature sensitive streptomycin protein. Proc. Nat. Acad. Sci. 65: 544-550.

Karamata, D. and Gross, J.D. 1970. Isolation and genetic analysis of temperature sensitive mutants of Bacillus subtilis defective in DNA synthesis. Molec. Gen. Genetics 108: 277-287.

Khesin, R.B., Mindlin, S.Z., Gorlenko, Z.M. and Ilyina, T.S. 1968. Temperature sensitive mutations affecting RNA synthesis in Escherichia coli. Molec. Gen. Genetics 103: 194-208.

Kohiyama, M., Cousin, D., Ryter, A. and Jacob, F. 1966. Mutants thermosensibles d'Escherichia coli I. Isolement et caractérisation rapide. Annales de l'Institut Pasteur 110: 465-486.

Kuwana, H. 1961. Studies on a temperature sensitive irreparable mutant of Neurospora crassa. II. Osmotic nature of the mutant b39a. Jap. J. Genetics 36: 187-199.

Lehman, I.R., Bessman, M.J., Simms, E.S. and Kornberg, A. 1958. Enzymatic synthesis of deoxyribonucleic acid. I. Preparation of substrates and partial purification of an enzyme from Escherichia coli. J. Biol. Chem. 233: 163-170.

Low, B., Gates, F., Goldstein, T. and Söll, D. 1971. Isolation and partial characterization of temperature sensitive Escherichia coli mutants with altered leucyl- and seryl-transfer ribonucleic acid synthetases. J. Bacteriol. 108: 742-750.

Maas, W.K. and Davis, B.D. 1952. Production of an altered pantothenate-synthesizing enzyme by a temperature sensitive mutant of Escherichia coli. Proc. Nat. Acad. Sci. 38: 785-797.

Marinus, M.G. and Adelberg, E.A. 1970. Vegetative replication and transfer replication of deoxyribonucleic acid in temperature sensitive mutants of Escherichia coli K12. J. Bacteriol. 104: 1266-1272.

McElroy, W.D. and Mitchell, H.K. 1946. Enzyme studies on a temperature sensitive mutant of Neurospora. Fed. Proc. 5: 376-379.

McFall, E. and Heincz, M.C. 1970. Thermosensitive regulation of D-serine deaminase synthesis in a mutant of Escherichia coli K12. Molec. Gen. Genetics 106: 371-377.

Mitchell, H.K. and Houlahan, M.B. 1946. Adenine-requiring mutants of Neurospora crassa. Fed. Proc. 5: 370-375.

Normark, S. 1971. Phenethyl alcohol as a suppressor of the envA phenotype associated with the envA gene in Escherichia coli K12. J. Bacteriol. 108: 51-58.

Normark, S. 1969. Mutation in Escherichia coli K12 mediating sphere-like envelopes and changed tolerance to ultraviolet irradiation and some antibiotics. J. Bacteriol. 98: 1274-1277.

Normark, S. and Westling, B. 1971. Nature of the penetration barrier in Escherichia coli K12: Effect of macromolecular inhibition on penetrability in strains containing the envA gene. J. Bacteriol. 108: 45-50.

Normark, S., Boman, H.G. and Matsson, E. 1969. Mutant of Escherichia coli with anomalous cell division and ability to decrease episomally and chromosomally mediated resistance to ampicillin and several other antibiotics. J. Bacteriol. 97: 1334-1342.

Printz, D.B. and Gross, S.R. 1967. An apparent relationship between mistranslation and an altered leucyl-tRNA synthetase in a conditional lethal mutant of Neurospora crassa. Genetics 55: 451-467.

Reeve, J.N., Groves, D.J. and Clark, D.J. 1970. Regulation of cell division in Escherichia coli: characterization of temperature sensitive division mutants. J. Bacteriol. 104: 1052-1064.

Reynolds, P.E. 1966. Antibiotics affecting cell-wall synthesis. p. 47-69. in Biochemical Studies of Antimicrobial Drugs, Sixteenth Symposium of the Society for General Microbiology, London, Cambridge University Press, 1966. 349 pages.

Ricard, M. and Hirota, Y. 1969. Effet des sels sur le processus de division cellulaire d'Escherichia coli. C. R. Acad. Sc. Paris 268: D-1335-1338.

Rogers, D. 1968. Osmotic pools in Escherichia coli. Science 159: 531-532.

Ron, E.Z. and Davis, B.D. 1971. Growth rate of Escherichia coli at elevated temperatures: Limitation by methionine. J. Bacteriol. 107: 391-396.

Russell, R.R.B. and Pittard, A.J. 1971a. New suppressor in Escherichia coli. J. Bacteriol. 107: 736-740.

Russell, R.R.B. and Pittard, A.J. 1971b. Mutants of Escherichia coli unable to make protein at 42C. J. Bacteriol. 108: 790-798.

Shapiro, B.M., Siccardi, A.G., Hirota, Y. and Jacob, F. 1970. On the process of cellular division in Escherichia coli II. Membrane protein alterations associated with mutations affecting the initiation of DNA synthesis. J. Mol. Biol. 52: 75-89.

Siccardi, A.G., Shapiro, B.M., Hirota, Y. and Jacob, F. 1971. On the process of cellular division in Escherichia coli IV. Altered protein composition and turnover of the membranes of thermosensitive mutants defective in chromosomal replication. J. Mol. Biol. 56: 475-490.

Smith, H.S. and Pardee, A.B. 1970. Accumulation of a protein required for division during the cell cycle of Escherichia coli. J. Bacteriol. 101: 901-909.

Steinberg, W. and Anagnostopoulos, C. 1971. Biochemical and genetic characterization of a temperature sensitive, tryptophanyl-transfer ribonucleic acid synthetase mutant of Bacillus subtilis. J. Bacteriol. 105: 6-19.

Streisinger, G., Mukai, F., Dreyer, W.J., Miller, B. and Horiuchi, S. 1961. Mutations affecting the lysozyme of phage T4. Cold Spring Harbour Symposium on Quantitative Biology 26: 25-30.

Suzuki, D.T. 1970. Temperature sensitive mutations in Drosophila melanogaster. Science 170: 695-706.

Thompson, L.H., Mankovitz, R., Baker, R.M., Till, J.E., Siminovitch, L. and Whitmore, G.F. 1970. Isolation of temperature sensitive mutants of L-cells. Proc. Nat. Acad. Sci. 66: 377-384.

Wainwright, R.R. and McVeigh, I. 1967. Requirements of two temperature sensitive mutant strains of Neurospora crassa for growth at elevated temperatures. Amer. J. Botany 54: 582-588.

Walker, J.R. and Smith, J.A. 1970. Cell division of the Escherichia coli lon$^-$ mutant. Molec. Gen. Genetics 108: 249-257.

Weigand, R.A., Shively, J.M. and Greenawalt, J.W. 1970. Formation and ultrastructure of extra membranes in Escherichia coli. J. Bacteriol. 102: 240-249.

Whitney, E.N. 1971. The tolC locus in Escherichia coli K12. Genetics 67: 39-53.

Worcel, A. 1970. Induction of chromosome re-initiations in a thermosensitive DNA mutant of Escherichia coli. J. Mol. Biol. 52: 371-386.

Yaniv, M. and Gros, F. 1969. Studies on valyl-tRNA synthetase and $tRNA^{val}$ from Escherichia coli III. Valyl-tRNA synthetases from thermosensitive mutants of Escherichia coli. J. Mol. Biol. 44: 31-45.