Characterization of a thermosensitive ribonucleotide reductase mutant derived from *Corynebacterium ammoniagenes* ATCC 6872 and its use in the production of nucleotides

Von dem Fachbereich Biologie

der **Universität Hannover**

zur Erlangung des Grades eines

Doktors de Naturwissenschaften

-Dr. rer. nat.-

genehmigte Dissertation

von

M.Sc. Hesham Elhariry

geboren am 27. 05. 1969

in Kairo, Ägypten

2004

Referent: Prof. Dr. G. Auling

Korreferent: Prof. Dr. H.-J. Jacobsen

Mitprüfer: Prof. Dr. A. Brakhage

Tag der Promotion: 10.05.2004

ABSTRACT

Hesham Elhariry

Characterization of a thermosensitive ribonucleotide reductase mutant derived from *Corynebacterium ammoniagenes* ATCC 6872 and its use in the production of nucleotides

Coryneform bacteria are widely used for the production of flavor enhancers and other nucleotides by direct fermentation of sugar into 5´-ribonucleotides. Here, the metabolic correlation between nucleotide accumulation and arrest of cell-cycle in the B-, C-, and D-phases was studied in non-synchronized cultures of *C. ammoniagenes* wild-type and a thermosensitive (ts) mutant derived therefrom. Particular emphasis was laid on the inhibition of DNA precursor biosynthesis in the wild-type by addition of radical scavengers or by heat treatment of the *nrd* (*n*ucleotide *red*uction) mutant CH31. Direct or indirect inhibition of the cell-cycle of the wild-type strain ATCC 6872 by addition of antibiotics or radical scavengers induced only limited elongation characteristic of corynebacteria. In the order of B-, C-, and D-phase, the earliest inhibition of the cell-cycle yielded the highest accumulation of NAD. The highest level (1.52 g / l) of NAD was accumulated when the cell-cycle of the ts-mutant CH31 was arrested by temperature shift to non-permissive conditions.

To identify the putative point mutation of the ts-mutant CH31 in the *nrdE* gene, 5.2 kb *XmaI*-fragment from the chromosomal DNA of the CH31 strain or its parent strain were cloned. Sequence comparison of the *nrdE* genes revealed a nucleotide exchange at position 1301 from cytosine to thymine. The deduced amino acid sequence of NrdE indicated an exchange in the position 434 resulting in the substitution of serine for phenylalanine adjacent to the active site. In order to determine the consequence of this amino acid exchange for the thermosensitivity of the ts-mutant CH31, either $nrdE^+$ or $nrdE^{ts}$ genes were cloned and expressed in this mutant. Introduction of the $nrdE^+$ gene from the wild-type but not from the mutant complemented the thermosensitive phenotype of strain CH31.

Under non-permissive conditions the strain CH31 was also able to accumulate IMP. Extracellular accumulation of either NAD or IMP was distinctly enhanced by adding precursors for exploitation of salvage pathways. For further improvement of IMP production the ts-mutant CH31 was grown in a 10-liter bioreactor under modified cultivation conditions.

Key words: *Corynebacterium ammoniagenes*, manganese-ribonucleotide reductase, cell cycle, limited elongation, scanning electron microscopy, 5´-IMP, NAD, flavor enhancer.

Zusamenfassung

Hesham Elhariry

Charakterisierung der thermosensitiven Ribonucleotid Reduktase Mutante von *Corynebacterium ammoniagenes* ATCC 6872 und deren Nutzung für die Produktion von Nucleotiden.

Coryneforme Bakterien werden in industriellem Maßstab für die Produktion von Geschmacksverstärkern und anderen Nucleotiden durch direkte fermentative Umsetzung von Zuckern zu 5´-Ribonucleotiden genutzt. In dieser Arbeit wurde die Beziehung zwischen der Anhäufung von Nucleotiden und der Unterbrechung des Zellzyklus in der B-, C- und D-Phase untersucht. Dazu wurden exponentielle Kulturen von *C. ammoniagenes* ATCC 6872 und der daraus hergestellten thermosensitiven Mutante CH31 hinsichtlich der Unterdrückung der DNA-Vorstufensynthese durch Zugabe von Radikalfängern oder durch Hitzebehandlung der *nrd* (*n*ucleotide *red*uction)-Mutante untersucht.

Direkte und indirekte Unterbrechung des Zellzyklus beim Wildtyp-Stamm ATCC 6872 durch Zugabe von Antibiotika oder Radikalfängern bewirkte nur eine eingeschränkte Verlängerung der Zellen, wie sie für Corynebakterien typisch ist. Die Hemmung zum frühest möglichen Zeitpunkt (B-Phase) führte zu einer höheren Produktion von NAD als eine Unterbrechung in der C- und D-Phase. Der höchste NAD-Wert (1,52 g / L) wurde bei Hemmung des Zellzyklus der *nrd*^{ts}-Mutante durch Erhöhung der Kultivierungstemperatur auf nicht-erlaubte Bedingungen erreicht (37°C).

Um die Punktmutation im *nrdE*-Gen der *nrd*-Mutante CH31 zu identifizieren, wurde ein 5,2 kb *Xma*I-Fragment aus der chromosomalen DNA des Stammes CH31 oder des Elternstammes kloniert. Ein Sequenzvergleich der *nrdE*-Gene ergab einen Nucleotid-Austausch von Thymin gegen Cytosin an Position 1301. Die abgeleitete Aminosäure-Sequenz des NrdE-Proteins zeigte an Position 434 einen Austausch von Serin gegen Phenylalanin in der Region neben dem katalytischen Zentrum. Um die Verantwortlichkeit dieser Punktveränderung festzustellen, wurden *nrdE*-Gene des Wildtyps und der Mutante in die *nrdE*^{ts} Mutante CH31 kloniert und dort exprimiert. Mit dem *nrdE*-Gen des Wildtyps konnte durch genetische Komplementation wieder Wildtyp-Verhalten erreicht werden, nicht jedoch mit dem defeken *nrdE*-Gen des Stammes CH31. Die Mutante CH31 konnte bei erhöhter Temperatur auch IMP akkumulieren. Sowohl die Produktion von NAD wie von IMP ließ sich durch Zugabe von Vorstufen im salvage pathway dramatisch steigern. Für die IMP-Produktion wurden die Ergebnisse aus Schüttelkolben erfolgreich auf die Anzucht im 10 L-Maßstab (Bioreaktor) übertragen.

Schlagworte: *Corynebacterium ammoniagenes*, Mangan-Ribonucleotid Reduktase, Zellzyklus, Rasterelektronenmikroskopie, 5´-IMP, NAD, Geschmacksverstärker

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

µ-	micro- (10^{-6})
A	absorbance
aa	amino acid
ADH	alcohol dehydrogenase
amp^r	ampicillin, ampicillin-resistance
APS	ammonium persulfate
ATCC	american type culture collection
BCIP	5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate) 4-toluidine salt
bp	base pair
BSA	bovine serum albumin
cf.	compare (L.*confer*)
Ci	curie, (3.7 x 10^{10} disintegrations per second)
cm	centimeter
Cm, *cm^r*	chloramphenicol, chloramphenicol resistance
CTAB	N-cetyl-N,N,N-trimethyl ammonium bromide
Da	dalton
DMF	dimethyl-formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene-di-amine- tetra-acetate
Fig.	figure
fts	*f*ilamentation *t*hermo*s*ensitive
h	hour
HEPES	N-2-Hydroxyethylpiperazine-N´-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
HU	hydroxyurea
IPTG	isopropyl-ß-D-thiogalactoside
J	joule
kb	kilo base
kDa	kilo Dalton
Km, *km^r*	kanamycin, kanamycin-resistance
l	liter
LB	luria Bertani
m	meter
M	molar

m-	milli- (10^{-3})
mA	milliampere
mer	polymer
min	minute
MNNG	*N*-methyl-*N*-nitro-*N*-nitrosoguanidine
Mn-RNR	manganese-containing RNR
MP	*p*-Methoxyphenol
MW	molecular weight
n-	nano- (10^{-9})
NAD^+	nicotinamide-adenine dinucleotide (oxidized form)
NBT	nitroblue tetrazoliumchloride
Nr.	number
nrd	*n*ucleotide *red*uction
nrdE	the gene encoding for the large catalytic subunit of RNR
NrdE	large catalytic subunit of the Mn-RNR (also known as R1E) encoded by *nrdE*
nrdE^{ts}	mutated *nrdE* from *Corynebacterium ammoniagenes* strain CH31
nrdF	the gene encoding for the small subunit (metallo-cofactor) of RNR
nt	nucleotide
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pO2	pressure of oxygen
R1	large catalytic subunit of the Fe-RNR encoded by *nrdA*
R1E	large catalytic subunit of the Mn-RNR (also known as NrdE) encoded by *nrdE*
R2F	small subunit (metallo-cofactor) of the Mn-RNR, encoded by *nrdF*
RBS	ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
RNR	ribonucleotide reductase
rpm	revolution per min
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
Sec.	section

TAE	tris-acetic acid- EDTA
T_{anneal}	annealing temperature
TE	tris-EDTA
TEMED	*N,N,N´,N´*-tetramethylethylenediamine
T_m	melting temperature
Tris	tris (hydroxymethyl) aminomethane
ts	thermosensitive
U	unit
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Nucleotides, Nucleosides, and Bases

dATP	2´-deoxyadinosine 5´-triphosphate
dCTP	2´-deoxycytidine 5´-triphosphate
dGTP	2´-deoxyguanisine 5´-triphosphate
dNTP	2´-deoxyribonucleotide 5´-triphosphate
dTTP	2´-deoxythymidine 5´-triphosphate
dUTP	2´-deoxyuridine 5´-triphosphate
GMP	guanosine 5´-monophosphate
IMP	inosine 5´-monophosphate
NTP	nucleotide 5´-triphosphate
XMP	xanthosine 5´-monophosphate

a, A	adenine
c, C	cytosine
g, G	guanine
t, T	thymine
Hx	hypoxanthine

1   INTRODUCTION

From a historical point of view, humans have practiced biotechnology for thousands of years, for the production of bread, beer and wine. Microorganisms are currently used to manufacture products for human and animal health care, food and agriculture, and environment pollution management. Consequently, specially selected microorganisms have been used to manufacture commodity and specialty chemicals. Commodity chemicals produced in large quantity at low cost are primary metabolites, such as ethanol and amino acids. Specialty chemicals such as nucleotides, vitamins and pharmaceuticals, are manufactured at a substantially higher cost (Lillehoj and Ford 2000).

Primary metabolites are the small molecules in living cells; they are intermediates or end products of the pathways of intermediary metabolism, building blocks for essential macromolecules, or are converted into coenzymes (Demain 2000). Modern biotechnology is concerned with the application of scientific techniques using living organisms, or substances from those organisms, to make or modify products, improve plants and animals, or to develop microorganisms for specific uses. Strain improvement and finding alternative methods are two of the important applications in biotechnology researches.

New knowledge, mainly gained in the last century of microbiology and biochemistry has revealed the importance of microorganisms for flavor development. As a consequence, much research has since been focused on the possibilities of designing processes for flavor production under well-understood and controlled conditions (Ogata *et al.* 1976). As a contrast to chemically produced substances, the natural flavors that produced by biological methods are favored by consumers who are increasingly concerned with possible health issues and environmental damage caused by synthetic chemicals and their production. For the bioproduction of flavor several approaches are possible: extraction from plant material, plant cell cultures, enzymatic synthesis or the use of specific microorganisms (Cheetham 1993; Vanderhaegen *et al.* 2003).

1.1 Production of flavor enhancers by corynebacteria

The perception of flavor is a combination of the sensations perceived by the two chemical senses, taste and smell. A considerable amount of work, especially by flavor companies, has been conducted into the production of flavor enhancers (Nagodawithana 1994). Deibler and Delwiche (2004) have reviewed generation of desirable flavors, and analysis of important flavor precursors in food. Flavor enhancers such as inosine monophosphate, and guanosine monophosphate are natural components of meat (Shi and Ho 1994). They have been shown to improve flavor and have been used by the Japanese for many years to give '*Umami*', a Japanese term meaning deliciousness. This trend has spread not only within Japan but also to other Asian countries, Europe and the United States, with the result that the consumption of these seasonings has increased rapidly. Recently, since '*Umami*' taste has been accepted world-wide as a new category of taste, many scientists have extensively investigated these seasonings in regard to such aspects as physiology, nutrition, food science and psychology (Kawamura and Kare 1987; Reineccius 1994). Among the nucleotides that have been shown to best enhance the taste of food are those that have a purine nucleus with a hydroxyl group in the 6´ position and a ribose, a moiety esterified in the 5´ position with phosphoric acid, such as inosine 5´-monophosphate (IMP), guanosine 5´-monophosphate (GMP), and xanthine 5´-monophosphate (XMP) (Nagodawithana 1994). By simple use, nucleotides improve the natural taste and flavor of meat, poultry, fish, vegetables and other prepared foods. They also help to moderate basic flavors: sweetness, sourness, saltiness and bitterness. Also, they suppress undesirable odors of food such as sulfurous and starchy and the typical smells associated with hydrolyzed vegetable proteins, as well as the "canned" or "processed" smells of preserves and frozen foods. The use of nucleotides in the food is approved by the FAO and WHO (http://www.leffingwell. com/ilsi.htm). Monosodium glutamate and the nucleotides IMP or GMP act synergistically. For example, a 1:1 mixture gives flavor intensity 30 times stronger than that of monosodium glutamate alone (Maga 1994).

In accordance with the use of the non-pathogenic coryneform bacteria in

the production of primary metabolites such as amino acids and nucleotides, the main focus in biochemical and molecular genetic work has been on their primary metabolites (Demain 2000). One of the important directions in the field of primary metabolite synthesis in corynebacteria is the production of the nucleotides IMP, GMP, and XMP as flavor enhancers in mutants of *Corynebacterium glutamicum* and *C. ammoniagenes*. These nucleotides can be produced by direct fermentation of sugars into 5'-ribonucleotides through *de novo* pathway or by salvage synthesis from precursors in the fermentation medium. The productive strains have been bred by the successive introduction of combinations of auxotrophism, analog resistance, cofactor-resistance and leaky mutations into suitable wild-type strains (Kuninaka 1986). Increasing the productivity of the initial isolates requires a program of genetic improvement. A mutagen, such as ultraviolet light, ionizing radiation, or a chemical agent, is applied and the mutant strains are screened under special growth conditions. This is usually an iterative process, mutant strains are screened, remutageneized, and reselected several times until a culture with commercial potential is obtained (Anderson 2000; Glick and Pasternak 2003). However, nucleotide accumulation was severely affected by very low concentrations of manganese ions (Mn^{2+}) and excessive amounts of Mn^{2+} (more than 20 µg per liter) drastically inhibited IMP-accumulation (Oka *et al.* 1968; Teshiba and Furuya 1982). For the industrial production of IMP, a series of stepwise mutants of *Corynebacterium ammoniagenes* has been selected (Teshiba and Furuya, 1983, 1984). These researchers isolated a Mn-insensitive mutant whose accumulation of 5′-IMP was not affected by the presence of high manganese ion concentration. Another profound alteration could be seen in a guanine auxotophic mutant. On the other hand, Mn^{2+} control of growth and DNA formation in *C. ammoniagenes* ATCC 6872 has been traced back to DNA precursor biosynthesis, i.e., manganese-dependent ribonucleotide reduction. Therefore, Mn^{2+}- limitation is a feasible approach for achieving perturbation of balanced growth of the Mn^{2+}-required coryneform bacteria resulting in nucleotide overproduction (Auling *et al.* 1980). However, it is difficult to adjust Mn^{2+}-concentrations in industrial fermentation media to precisely below a threshold value of 1 µM. Since *C. ammoniagenes* ATCC 6872 is sensitive to radical scavengers (Auling and Follmann 1994), hydroxyurea (HU) and *p*-methoxyphenol MP were applied as a novel process for nu-

cleotide production (NAD$^+$) in the presence of excessive amount of Mn^{2+} (Abbouni *et al.* 2003). Novel processes are available based on fermentation of sugars into nucleosides, with subsequent phosphorylation to the corresponding 5'-ribonucleotides (Mori *et al.* 1997; Asano *et al.* 1999; Mihara *et al.* 2000). In addition, purine nucleosides are commercially produced in bulk quantities by fermentation with *Bacillus sp* (Kuninaka, 1996; van Loon *et al.* 1996). Recently, the industry's efforts have been in the areas of analytical and synthetic chemistry, biotechnology, aroma component measurements, and addressing flavor problems of functional foods (Noguchi *et al.* 2003). Other nucleotides such as AMP, ATP, NAD, FAD, COA, *etc* are also prepared by fermentation to be pharmaceutical agents, although the amount of their production are not as large as that of the IMP and GMP.

Microorganisms synthesize the metabolites necessary for their growth by strong metabolic regulation and preventation of overproduction (Auling and Follmann 1994). Often, microorganisms are used for industrial production of numerous biochemicals under repressed cell-cycle. The advantages are increase in product yield due to decrease of energy loss for cell growth and decrease of by-product formation generally concomitant with cell growth. However, the molecular biology of the cell division process is poorly understood for coryneform bacteria (Kobayashi *et al.* 1997).

1.2 Prokaryotic cell-cycle

The history of bacterial growth and cell-cycle studies can be traced back almost 100 years. Most bacteria and archaea divide symmetrically in a process that is subject to extensive regulation to ensure that both newly formed daughter cells contain a copy of the chromosome. Bacterial cell division is very complex involving several stages that are currently not well understood. There is evidence to suggest that it involves multiple factors that are coordinated to interact precisely with one another. For instance, it appears that the complex processes of DNA replication, transcription, translation, cytokinesis, and chromosome partitioning are interdependent and precisely coordinated during cell division (Koch 2001). Most knowledge about the regulation of bacterial cell division and replica-

tion of DNA stems from the analysis of only three species, *Escherichia coli* (Vicente *et al.* 1998; Zhou *et al.* 1997), *Caulobacter crescentus* (Sackett *et al.* 1998; Jensen *et al.* 2001), and *Bacillus subtilis* (Rowland *et al.* 1997; Moriya 1999). Although other bacteria such as corynebacteria and Streptococci have important applications, although, these organisms have been used to study specific aspects of metabolism or cell division or to emphasize differences or similarities of interest. When other bacteria were studied it has been within the framework of the *E. coli* model of the division cycle.

Generally, the cell division of prokaryotes can be broken down into a simple cycle, consisting of three phases, B, C, and D (Fig. 1.1). Continuous growth (B-period) triggers DNA replication and this period is not seen at moderate or high growth rates. DNA replication (C-period) proceeds until the completion of chromosome replication and the first signs of septation are visualized after the termination of chromosomal replication, the segregation of the nucleoid and the initiation of septum formation. The time between termination of DNA replication and cell division is known as the D-period, the actual division of the cell and fission of the cytoplasm. The important point is that complex division cycles are due to the overlapping of the simpler B+C+D sequences. Under laboratory conditions using defined growth media adjustments with respect to protein synthesis, RNA synthesis, DNA synthesis, and cell size have been studied extensively for *E. coli*. Briefly, at faster growth cells become bigger, reflecting the increased genome content per cell. It has been shown that the start of DNA replication is directly correlated with cell size. This size has been termed initiation mass, and division occurs during a fixed period after initiation of DNA replication. In this way, the rate of division adjusts to growth conditions (Cooper 1991; Ayala *et al.* 1994). DNA supercoiling is important in the compaction of DNA into the nucleoid, as well as the regulation of gene expression and DNA replication. This compaction of the chromosome into the nucleoid and its dynamic behavior during partitioning of the DNA has long been known to constitute a vital stage of cell division. Generally, bacteria contain two different type II topoisomerases: DNA gyrase and topoisomerase IV. Gyrase is unique among topoisomerases, in that it is the only known enzyme that can actively underwind (*i.e.*, negatively supercoil) DNA.

DNA gyrase works primarily in conjunction with topoisomerase I to bring about the superhelical density of DNA in bacteria. Gyrase also plays important roles in the initiation of DNA replication and elongation of DNA strands. Topoisomerase IV is required for proper chromosome segregation and the removal of knots in the genetic material (Woldringh and Odijk 1999).
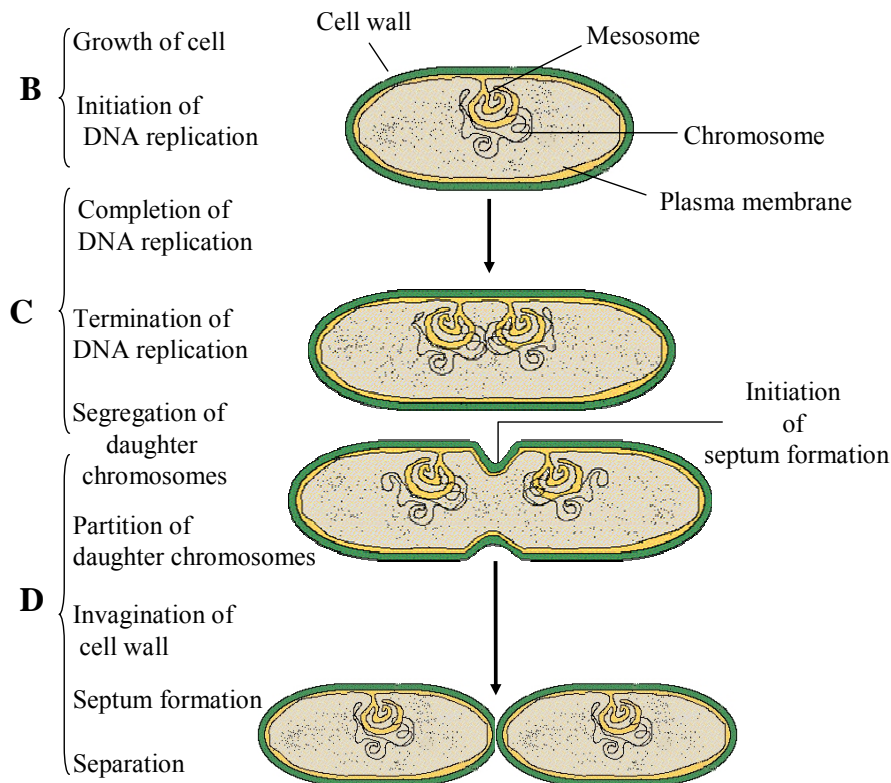
Fig. 1.1 Bacterial division cycle model**.** The key processes and the shape of cells due to overlapping of the simple B-, C-, and D-period are shown (Lengeler *et al.* 1999). A diagram of the attachment of bacterial chromosomes, indicating the possible role of the mesosome (an inward fold of the cell membrane) in ensuring the distribution of the "chromosomes" in a dividing cell. Upon attachment to the plasma membrane, the DNA replicates and reattaches at separate points (for more details see, http://home.earthlink.net/~dayvdanls/pcelldiv.htm).

In *E. coli*, numerous cell division genes have been identified in *E. coli* temperature sensitive mutants cells that did not divide properly, and hence were called *fts* genes, for *f*ilamentation *t*emperature *s*ensitive. Currently, all identified cell di-

vision gene products have been localized at the site of cell division (Lutkenhaus and Addinall 1997).

Genetic and microscopic studies have provided clues about the sequence of events occurring during division. The *ftsZ* gene is essential for cell division in bacteria that divide by binary fission, such as *E. coli* and *B. subtilis* (Beall and Lutkenhaus 1991; Dai and Lutkenhaus 1991), whereas in *Streptomyces, ftsZ* is required only for septation during sporulation (McCormick *et al.* 1994). In *B. subtilis*, it is required not only for vegetative cell division but also for asymmetric division during sporulation (Beall and Lutkenhaus 1991). A large number of genes is involved in the cell division process. The list of these genes includes *ftsA, ftsL, ftsI, ftsQ, ftsN, ftsW, ftsK,* and *ftsZ*. The products of these genes are involved in the cytoplasmic and periplasmic stages of peptidoglycan biosynthesis and cell septation. Among them the product of *ftsI* is the penicillin binding protein synthetase (known as PBP3 or FtsI). The FtsI is a peptidoglycan biosynthetic enzyme that is required for the formation of the peptidoglycan layer of the division septum. Concerning the division process, it is clear that in the actively growing periplasmic peptidoglycan layer is matched by a cytoplasmic FtsZ ring of continuously decreasing diameter. FtsZ, which binds GTP and has a GTPase activity, plays a central role in cytokinesis as a major component of the contractile ring. The assembly of the FtsZ ring at midcell occurs well before constriction is initiated. In addition to *ftsZ*, many more genes are specifically involved in cell division. The other cell division proteins are later recruited to the FtsZ ring to form the membrane-associated septal ring that mediates septation (Nanninga 2000). The role of the Min (minicell) proteins in cell division and assessment of the different strategies used by two rod-shaped bacteria is shown in Fig. 1.2, Gram-negative *E. coli* and the Gram-positive *Bacillus subtilis*. For the Gram negative model organism *E. coli*, the minicell genetic locus has three genes: *minC, minD* and *minE*. Deletion of *minC*, and *minD* or all three *min* genes gives rise to a minicell phenotype, whereas inactivation of the *minE* gene alone stimulates the formation of long filamentous cells. When the ratio of MinD to MinE is normal, the division inhibitory activity of MinCD is restricted to the potential division sites (PDSs) leaving the midcell PDS free for septum formation. Therefore, MinE would impact the topological

specificity, ensuring medial cell division (Rothfield and Zhao 1996; Pichoff *et al.* 1997; Rothfield *et al.* 1999).

The Gram-positive model organism *B. subtilis*, has homologues of MinC and MinD, however, its genome lacks a *minE* (Fig. 1.2). The topological specificity of the MinCD division inhibitor in *B. subtilis* appears to be mediated by the product of the unrelated *divIVA* gene (Cha and Stewart 1997; Edwards and Errington 1997). After completion of DNA replication, a new potential division site is created at midcell.
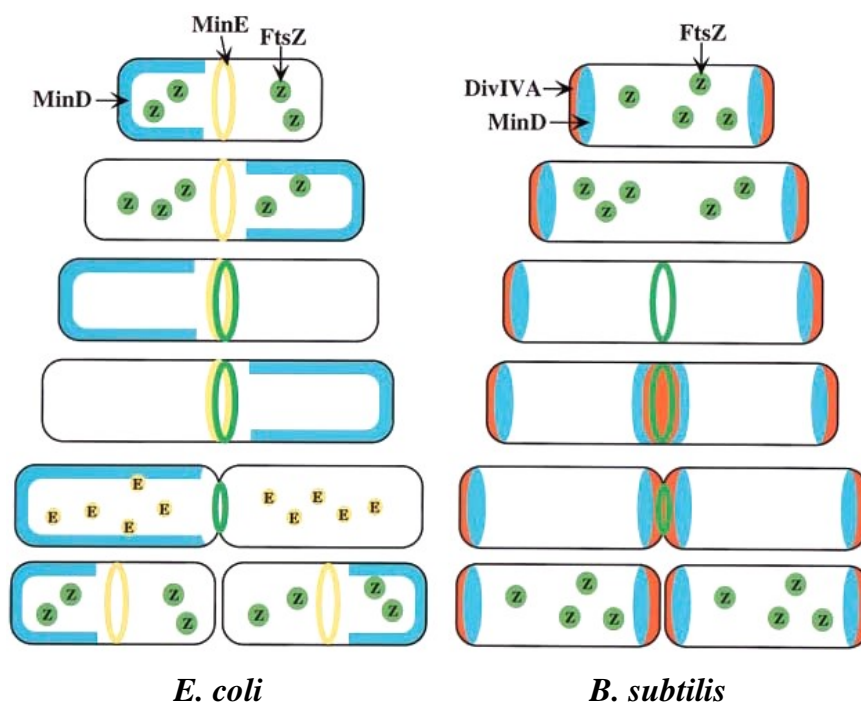
Fig. 1.2 Models for division-site selection in *E. coli* (*Left*) and *B. subtilis* (*Right*). MinD is in blue, MinE in yellow, FtsZ in green and DivIV in red. Different stages of the cell-cycle were shown, beginning with a newborn cell and finishing with cell division that produces two daughter cells (Jacobs and Shapiro 1999).

The sequestration of the MinD inhibitor to the poles allows the assembly of the FtsZ ring at midcell and the recruitment of other cell division proteins. At this point, the division machinery presumably becomes resistant to MinCD inhibition, perhaps because the presence of other cell division proteins stabilizes the FtsZ ring (Levin *et al.* 1998). DivIVA is then recruited to midcell, possibly by a later synthesis division protein. Assembly of DivIVA promotes the targeting of some

MinD proteins to midcell. Constriction is then initiated. When constriction is completed, the division apparatus disassembles, but DivIVA and MinD remain at the newly formed poles. Thus, both daughter cells have MinD inhibitor at their poles, preventing further divisions from taking place at these polar sites (Jacobs and Shapiro 1999). More recently, Ramos *et al.* (2003b) have stated that DivIVA may be important in regulating the apical growth of daughter cells during the cell-cycle of the rod-shaped actinomycete *Brevibacterium lactofermentum*.

Because of the cellular basis of life, its maintenance and its proliferation require the multiplication of cells. For the individual cell this is achieved by fission of a cell that is on average two times as large as a newborn one. All macro-molecular building blocks must be duplicated before fission. Clearly, the most important components of a cell to be replicated is its DNA. Purine and pyrimidine nucleotides make up the monomeric units of RNA and DNA; RNA synthesis is required for protein synthesis and DNA synthesis is required for growth and cell division (Kronberg 1974). Moreover, the nucleotides are physiological mediators in a number of cellular metabolic processes and play a major role in structural, energetic and regulatory functions. Purines and pyrimidines can be formed by *de novo* biosynthesis or salvage of preformed bases and interconversion to the desired compound (Kulkarni *et al.* 1998). Whether the nucleotides are derived from *de novo* biosynthetic pathway or from the salvage of preformed extracellular nucleobases, their formation is completely dependent on the synthesis of the precursor 5-phospho-D-ribosyl-α,1-pyrophosphate (PRPP), which is catalyzed by the enzyme PRPP synthetase. The pathway for *de novo* synthesis proceeds in two stages: synthesis of IMP (inosine 5´-monophosphate), which contains the base hypoxanthine, in 11 steps, and the four-step conversions of IMP to ATP and IMP to GTP (Switzer *et al.* 2002). Subsequently, the formation of purine and pyrimidine deoxyribonucleotides from ribonucleotides is catalyzed by ribonucleotide reductase (RNR), the only enzyme that converts the ribonucleotides to their deoxyribonucleotides forms using diverse metallo-cofactors in a radical mechanism (Fig. 1.3a). Despite these enzymes central role in nucleic acid metabolism neither their primary or quaternary structures nor their cofactor requirements have been evolutionarily conserved. The metallo-cofactors include a diferric tyrosyl radical (Class

I), adenosylcobalamin (Class II) and a glycyl radical (Class III). A fourth class (Class IV) is proposed to contain manganese and a tyrosyl radical (Fig. 1.3b).
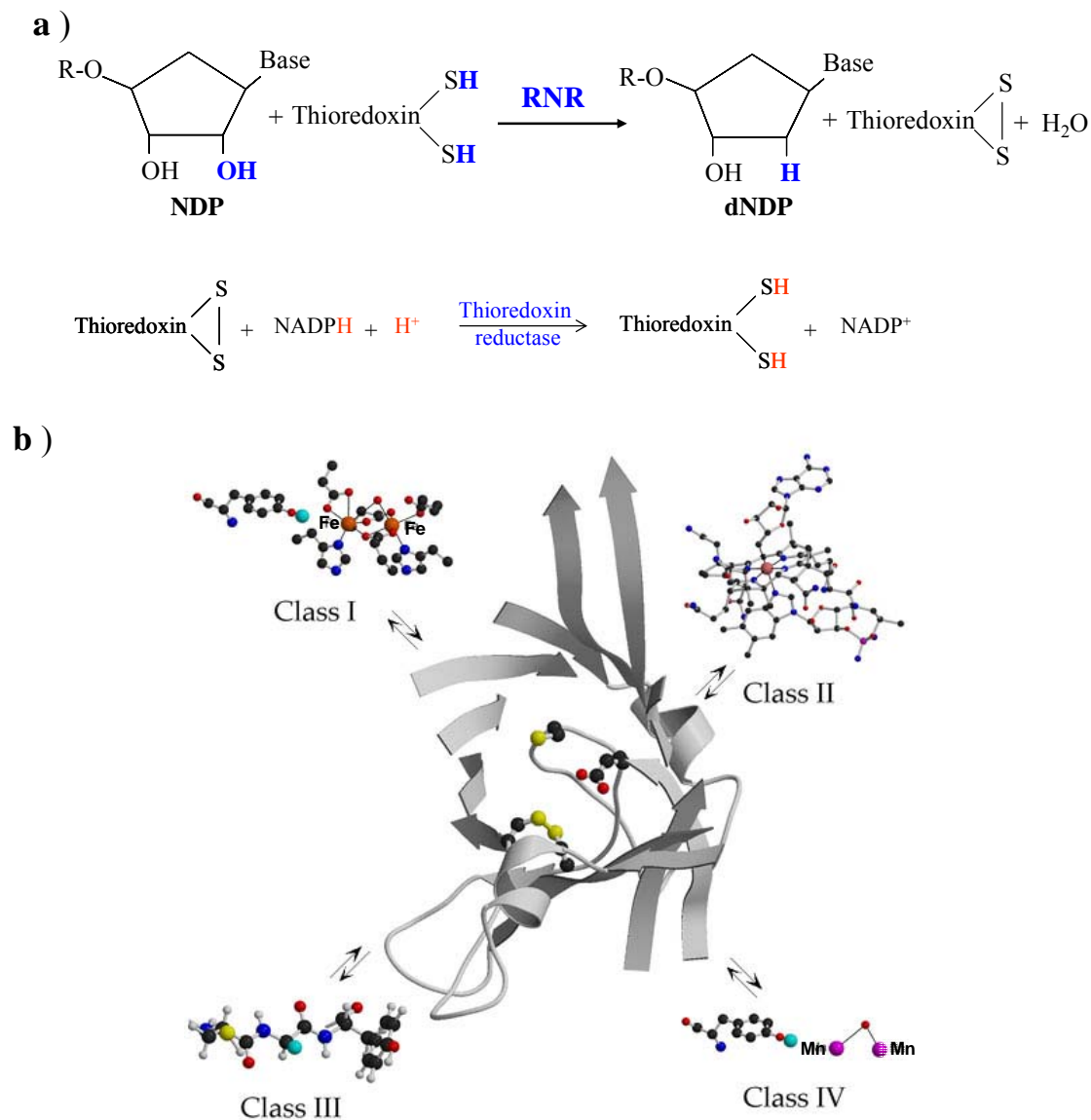
Fig. 1.3 Reduction mechanism of ribonucleotides (a) to the corresponding deoxyribonucleotides catalyzed by ribonucleotide reductase (RNR), R = PPi or PPPi (modified after Stubbe 1990). Classification of ribonucleotide reductases (RNRs) according to their metalocofactor (b) was also shown (Stubbe and van der Donk 1998).

The activity of RNR and its substrate specificity must be tightly regulated to ensure balanced production of all four of the dNTPs required for DNA replication. Such regulation occurs through the binding of nucleoside triphosphate effectors to either the activity sites or the specificity sites of the enzyme complex. The activity sites bind either ATP or dATP with low affinity, whereas the specificity

sites bind ATP, dATP, dGTP, or dTTP with high affinity. The binding of ATP at activity sites leads to increased enzyme activity, while the binding of dATP inhibits the enzyme (Stubbe and van der Donk 1995 &1998). Under conditions of the balanced growth (cf. Discussion), microorganisms synthesize the metabolites necessary for their growth and sustenance making use of strong metabolic regulation and preventing overproduction (Auling and Follmann 1994). The genetic organization for the purine nucleotide biosynthesis is well known in *E. coli* and *B. subtilis*. The *de novo* biosynthesis is regulated at the gene level and at the enzyme level at least in *E. coli* (White 2000), *B. subtilis* (Switzer *et al.* 2002).

1.3 Cell-cycle of coryneform bacteria

Corynebacteria are pleomorphic, asporogenous gram-positive bacteria widely distributed in nature. They are defined as irregular, non-sporulating, gram-positive rods. Their closest relatives are the genera *Mycobacterium, Nocardia, and Rhodococcus.* The non-pathogenic species of coryneform bacteria; such as *Corynebacterium glutamicum* (Shiio and Nakamori 1970; Pühler and Tauch 2003), *Corynebacterium ammoniagenes* (Teshiba and Furuya 1989), *Corynebacterium efficiens* (Fudou *et al.* 2002; Nishio *et al.* 2003), *Brevibacterium flavum, Brevibacterium lactofermentum* (González *et al.* 1996; Ramos *et al.* 2003b) are cost-effective bioconverters that are used for the industrial production of numerous biochemicals.

Coryneform bacteria have a unique mode of cell division, called post-fission snapping. They have thick cell walls consisting of two layers. The inner layer invaginates to form the septum and the outer layer breaks after cell division, resulting in a post-fission movement and the cell arrangements characteristics of these bacteria. However, the molecular biology of the cell division process is poorly understood for coryneform bacteria (Kobayashi *et al.* 1997). As a first step towards characterizing the cell division mechanism of coryneform bacteria, these authors cloned and sequenced the *ftsZ* gene from *Brevibacterium flavum*. They state that expression of *Br. flavum ftsZ* in *E. coli* inhibited its cell division, leading to filamentation. This demonstrated that the *Br. flavum ftsZ* product competed

with the *E. coli ftsZ* product. Moreover, Honrubia *et al.* (1998) identified and characterized the *ftsZ* gene from *Br. lactofermentum*. They found that the *ftsZ* gene from this organism does not complement *ftsZ* mutation or deletion in *E. coli*. Kijima *et al.* (1998) isolated *ftsZ* temperature sensitive (ts) mutants from *C. glutamicum*. The morphology of the ts-mutants of *C. glutamicum* remained normal when they were grown at 30°C. At the restrictive temperature (37°C) most of the ts-mutants formed elongated, club-shaped or dumbbell-shaped rods, however, they did not form filamentous cells. Moreover, these authors inhibited cell division using cephalexin and sparfloxacin, which are the inhibitors of septation and DNA synthesis respectively, and are known to cause cell filamentation in *E. coli*. These two antibiotics did not cause filamentation in *C. glutamicum* but induced morphological changes that were similar to those observed with the temperature sensitive mutants of *C. glutamicum*. In *Brevibacterium lactofermentum*, as in many Gram-positive bacteria, a *divIVA* gene is located downstream of the *dcw* cluster of cell-division and cell-wall-related genes (Ramos *et al.* 2003b). These authors suggested that the *divIVA* gene is needed for cell viability in *Br. lactofermentum*. DivIVA is also an essential protein localized to the ends of corynebacteria cells.

Rationale

The aim of this work was to elaborate fundamentals for the development of alternative nucleotide production by *Corynebacterium ammoniagenes*. Unbalanced growth (cf. Discussion) induced by manganese-deficiency was exploited in an early approach for accumulation of IMP (Oka *et al.* 1968) or NAD (Nakayama *et al.* 1968). Here, the metabolic correlation between accumulation of the nucleotides and arrest of division cycle (B-, C-, and D-phase) was studied in *C. ammoniagenes* wild-type and a thermosensitive (ts) mutant derived thereof. Particular emphasis was laid on the inhibition of DNA precursor biosynthesis.

The *nrd* (*n*ucleotide *red*uction) mutant CH31 was generated by random chemical mutagenesis with MNNG and its temperature sensitive phenotype was biochemically allocated to the large subunit (R1E protein) of ribonucleotide reductase (Luo *et al.* 1997). In order to confirm the location of the putative point

mutation the *nrdE^{ts}* gene of the ts-mutant was to be cloned and sequenced for comparison with the wild-type gene. Due to either incomplete (Oehlmann, 1998) or inconsistent alignment with the published (Fieschi *et al.* 1998) sequence of the *nrdE^{+}* gene additional cloning and sequencing of the latter from the parent strain of the ts-mutant was necessary. It was attractive to exploit this biochemically and genetically characterized mutant for an alternative process of IMP accumulation. For further enhancement of nucleotide production salvage pathway of the purine nucleotides may be exploited as well.

2   MATERIALS AND METHODS

2.1 Chemicals and enzymes

All chemicals which were not mentioned here are products of the company E. Merk, Darmstadt, Germany.

Amersham Buchler GmbH & Co. KG, Braunschweig

(5-3H) CDP, ammonium salt (0.37- 1.1 TBq / mmol, 10-30 Ci / mmol)

Boehringer Mannheim GmbH, Mannheim

Alkalaine-phosphatase, 108 154

BIOMOL Feinchemikalien GmbH, Hamburg, Germany:

Agarose (DNA grade), 50148

Albumin bovine (BSA), Fraction V (lyophilized), 01400

Nitroblue tetrazolium chloride (NBT), 06428

Sodium dodecyl sulfate (SDS), ultra pure (2 x cryst.), 51430

TRIS, Tris ultra pure (hydroxymethyl) amino methane, 08003

Bio-Rad Laboratories GmbH, Munich, Germany:

Protein standard, #161-0372

BioScience

Rb-anti-CEDAF # 2778, code 2092778

Carl Roth GmbH + Co., Karlsruhe, Germany:

Roti ®- Phenol for separation of DNA/RNA, 0038.2

Difco Laboratories, Augsburg, Germany:

Bacto agar, 0140-01

Brain heart infusion, 0037-17-8

Nutrient broth, 0003-17-8

Trypton peptone, 0123.17

Yeast extract, 0127-01-7

Fluka Chemie GmbH, Buchs, Germany:

Adenine (6-Aminopurine), 01830

Sodium N-lauroylsarcosinate solution, 61747

New England BioLabs GmbH, Frankfurt am Main, Germany:

1-kb DNA ladder, #N3232S

Lambda DNA-*Hin*dIII digested, #N3012S

T4 DNA ligase, #M0202S

T4-ligase buffer with 10 mM ATP, B0202S

ICN Biomedicals

Cephalexin, 150585

Perkin Elmer, Norwalk, CT, USA:

BigDye terminator cycle sequencing kit, 4303152

Qiagen GmbH, Hilden, Germany

QIAEX II gel extraction kit (500), 20051

Taq PCR Core Kit (250 units), 201223

Roche Diagnostics GmbH, Mannheim, Germany:

Anti-digoxigenin-AP, Fab fragments, from sheep, 1 093 274

Blocking reagent for nucleic acid hybridization and detection, 1 096 176

DNA molecular weight marker II, digoxigenin-labeled, 1 218 590

RNase A, 109 142

Shrimp alkaline phosphatase (SAP), 1 758 250

Serva Feinbiochemica GmbH, Heidelberg, Germany:

Citric acid, analytical grade, 13375

Ethidium bromide, 21238

Glycerol (plant), 23176

Novobiocin sodium salt, 30995

Tween $^®$ 80, pure, 37475

Sigma-Aldrich Chemie GmbH, Deisenhof, Germay:

Alcohol dehydrogenase, A-7011

Ampicillin (D-α- Aminobenzyllepenicillin) sodium salt, A-9518

Corn steep liquor (50% solids), C-4648

Glycerol, G-7757

Hydroxyurea, H-8627

Hypothanthine (6-Hydroxypurine), H-9377

Inosine 5´ monophosphate ($C_{10}H_{11}N_4O_8PNa_2$; 25.9% H_2O), I-4625

p-Methoxyphenol, M-5262

Nicotinic acid (Niacin; Pyridine-3.carboxylic acid), N-4126

D-Sorbitol, S-6021

2.2   Microorganisms, plasmids, and primers

Corynebacterium ammoniagenes ATCC 6872 and the ts-mutant CH31 were maintained in the culture collection, Institute of Microbiology, University of Hannover, Germany.

Table 2.1 Microorganisms and plasmids

Microorganisms	Related geno-/phenotype	Source/Reference
Corynebacterium ammoniagenes :		
ATCC 6872	Wild-type, *nrdE*$^+$	ATCC
CH31	Thermosensitive mutant of ATCC 6872, *nrdE*^{ts}	Luo *et al.* 1997
Escherichia coli:		
XL1-Blue	*end*A1, *gyr*A96, *hsd*R17 (r$_k^-$ m$_k^+$), *rec*A1, *rel*A1, *sup*E44, *thi*-1, F'(*pro*AB, lac1^{q}ZΔM15, Tn10)	Stratagene
Top10 F	F′ {*lac*Iq Tn*10* (TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen

Plasmids	Feature	Source/Reference
pUC18	*amp*^{r}, cloning vector	Yanisch-Perron *et al.* 1985
pUCEF6872	pUC18 with a 5.2 kb *Xma*I fragment from *C. ammoniagenes* ATCC 6872	This work
pUCECH31	pUC18 with a 2.16 kb *Bam*HI-*Sph*I fragment from the ts-mutant CH31	This work
pUCEFCH31	pUC18 with a 5.2 kb *Xma*I fragment from the *nrdE*^{ts} mutant CH31	This work
pCR$^®$2.1TOPO	*amp*^{r} and *km*^{r}	Invitrogen
pTopCH31	pCR$^®$2.1TOPO with a *Xba*I-*Sac*I PCR fragment containing *nrdE* from ts-mutant CH31	This work
pXMJ19	*cm*^{r}, *C. glutamicum* / *E. coli* shuttle vector containing remarks; p*tac*, *lac*Iq.	Jakoby *et al.* 1999
pXE6872	pXMJ19 with a *Xba*I-*Sac*I containing *nrdE* from *C. ammoniagenes* ATCC 6872	This work
pXECH31	pXMJ19 with a *Xba*I-*Sac*I fragment from pTopCH31	This work

The oligonucleotide primers used in this study for sequencing and amplification of *nrdE* gene were obtained from MWG Biotech AG, Germany (Table 2.2)

Table 2.2 Sequencing, and expression primers used in this study

Designation	Length-mer	GC-content%	Tm (°C)	Sequence (5´→ 3´)
CAE1 [F]	21	57.1	61.8	cgccgtgattaccatgaggtc
CAE2 [F]	22	45.5	58.4	aagtactacacttcctcaccc
CAE3 [F]	21	52.4	59.8	aacgctcaccacccagatatc
CAE4 [F]	17	64.7	57.6	ccgttgctgagctgcag
CAE5 [F]	21	66.7	65.7	ggccagagaacctccacggc
CAE6 [F]	22	45.5	58.4	cgatctcctacacaacaactc
CAE7 [R]	21	57.1	61.8	ttgtccatgtgtggagctggg
CAE8 [R]	20	50	61.8	atcttgttggaagcacgcag
CAE9 [R]	16	68.8	56.9	cgaccgtgccaccgaa
CAE10 [R]	21	47.6	57.9	acatgatgtatgggtaaccgg
CAE11 [R]	17	64.7	57.6	cgcacccagctggttag
CAE12 [R]	21	47.6	57.9	agtgtagtacttgtaggcacc
CAE13 [R]	21	61.9	63.7	ggctttcggctatcttcgcgc
XbCaE [F E]	36	50	71.7	ggggtctagatt-gaaaggccgagtgcttcaaatg ac
SaCaE [R E]	34	52.9	71.9	aaaggagctcttagagcatgcag-gagacgcaacc
OCA2-1 [F]	23	47.8	60.6	ccaacattgccttcatggaatcc
OCA2-4 [R]	20	60	61.4	gggaagagaccttcgtagcc

[F] Forward primer   [R] Reverse primer   [E] Expression primer.

2.3 Media

To prepare agar plates and slants, Difco-agar was added at level of 15 g / l and 20 g / l, respectively. If necessary, the pH of the used media was adjusted with 3 N NaOH. Generally, all media were autoclaved at 121°C for 20 min.

Seed medium (Nara *et al.* 1969)
D-glucose monohydrate	20	g
Pepton from casine	10	g
Yeast extract	10	g
NaCl	2.5	g
Deionized water up to	1	l

pH was adjusted at 7.2 before sterilization

Luria Bertani (LB) medium (Miller 1972)
Trypton peptone	10	g
Yeast extract	5	g
NaCl	5	g
Deionized water up to	1	l

pH was adjusted at 7.4 before sterilization

LBG medium (modified after Miller 1972)
Trypton peptone	10 g
Yeast extract	5 g
NaCl	5 g
Glucose	5 g
Deionized water up to	1 l

pH was adjusted at 7.4 before sterilization

Brain heart infusion (BHI) broth (Torrents *et al.* 2003)
Brain heart infusion (Difco)	37 g
Deionized water up to	1 l

NBH-medium (Auling 1980)
Nutrient broth	8 g
Yeast extract	2 g
NaCl	5 g
Deionized water up to	1 l

Fermentation medium for NAD production (modified after Nakayama *et al.* 1968)

D-glucose	100 g	
KH_2PO_4	10 g	
K_2HPO_4 x 3 H_2O	13.1 g	
$MgSO_4$ x 7 H_2O	10 g	
Urea	6 g	(autoclaved separately)
Yeast extract	10 g	
DL-calcium pantothenate	12 mg	(sterilized by filtration)
Thiamine hydrochloride	10 mg	(sterilized by filtration)
D(+) Biotin	30 µg	(sterilized by filtration)
Deionized water up to	1 l	

  pH was adjusted at 7.4 before sterilization

Fermentation medium for IMP production (Nakayama *et al.* 1968 and modified after Teshiba and Furuya 1982)

D-glucose	130 g	
KH_2PO_4	10 g	
K_2HPO_4 x 3 H_2O	13.1 g	
$MgSO_4$ x 7 H_2O	10 g	(Add after pH adjustment)
$ZnSO_4$ x 7 H_2O	1 mg	
Urea	6 g	(separately sterilized)
Yeast extract	10 g	
Nicotinic acid	5 mg	
DL-calcium pantothenate	10 mg	(sterilized by filtration)
Thiamine hydrochloride	5 mg	(sterilized by filtration)
D(+) Biotin	30 µg	(sterilized by filtration)
Deionized water up to	1 l	

  pH was adjusted at 8.3 before sterilization

For NAD^{+} or IMP production in the presence of high manganese ion concentration 10 µM of $MnCl_2$ x 4 H_2O was added as indicated in the text.

2.4 Microbiological methods

2.4.1 Maintenance of strains

The bacterial strains used in this study were stored for a long-term (several years) in media containing glycerol at low temperature without significant loss of their viability (Sambrook *et al.* 1989). Glycerol stocks were prepared by thoroughly mixing 0.8 ml of the overnight culture to a sterile screw-capped vial containing 0.7 ml of 87% sterile glycerol followed by shock freezing in liquid nitrogen and storing at -70°C. Viable bacteria were recovered by simply scratching the surface of the frozen stock with a sterile platinum wire. Bacterial strains were cultivated on seed or NBH slant agar, for 2 days at 27°C. The cultures that were needed for short-term use were kept at 4°C and were subcultured every 4-5 week.

2.4.2 Examination of ts-mutant CH31 phenotype

Before using the ts-mutant CH31 in the different applications, stability of the CH31 strain as a thermosensitive mutant was checked on seed agar plates at both 27°C and 37°C for 2 days. Single colonies grown only at 27°C were isolated and used in the further works.

2.4.3 Bacterial growth

2.4.3.1 *Measurement of turbidity*

As a rapid method to measure the bacterial growth, turbidity of the culture was measured at wave length 578 nm using a photometer model 1101 M (Eppendorf Gerätebau Netheter & Hinz GmbH, Hamburg, Germany). In order to determine the OD in the seed culture, 1ml of corynebacterial culture was centrifuged in eppendorf centrifuge at 8000 rpm for 5 min. The pellet was resuspended in 1 ml distilled water and subsequently diluted in water if necessary. To dissolve the salt crystals before determination of the OD in the fermen-

tation medium 1 ml of fermented culture was mixed with 3 ml distilled water and 100 µl 2N HCl. As the cells of *C. ammoniagenes* ATCC 6872 tend to adhere together 5 µl Tween 80 (20% w/v) were added and gently dispersed for 30 s in ultrasonic bath (Sonorex RK 102 H, Bandelin). The mixture was centrifuged at low-speed and the pellet was resuspended in distilled water. The turbidity was determined, and recalculated to cell dry weight using a standard curve.

2.4.3.2   Measurement of cell dry weight

The dry weight of cells was determined according to the method described by Beuse (1998) with some modifications. One volume of bacterial culture was mixed with three volumes distilled water in the presence of 100 µl 2N HCl (Sec. 2.4.3.1). The mixture was filtrated through membrane filter (OE 66, Scheicher and Schüll, Dassel, Germany) with 50 mm diameter and 0.2 µm pore size. The membrane which carried the cells was dried in a microwave oven with eight drying cycles, 2x at 250 watt for 2 min, 3x at 440 watt for 2 min, and 3x at 660 watt for 2 min.

After drying the membranes were cooled in a dissector for 10 min. The dry weight of cells was calculated as following:

$$DCW = \frac{\Delta DCW + \Delta m_F}{Volume\ of\ sample}$$

Where, DCW = dry cell weight, ΔDCW = the difference between the weight of the filter carring the cells (after drying) and the weight of empty filter, Δm_F = the loss of filter weight during drying (average of 5 empty filters)

A standard curve was created by plotting the obtained DCW values against the measured OD_{578} of the same samples. The obtained standard curve was used in the further work to calculate the dry weight of cells.

2.4.3.3   *Viable count*

Total viable cells were determined by standard microbiological methods and expressed as colony forming units (cfu). The withdrawn cell suspension was treated as described in Sec. 2.4.3.1 to dissolve salt crystals and separate the adhered cells, under sterile conditions. 0.1 ml of the desired dilution was spread over the surface of seed agar plates using a sterile glass spreader. The plates were incubated at 27°C for 2-3 days. Grown colonies were counted and cfu were calculated.

2.4.4 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of antibiotics was determined by the agar dilution method described by Willi *et al.* (1997). Serial dilutions of the examined antibiotics were prepared and incorporated into the growth medium (seed medium). Cultures of *C. ammoniagenes* ATCC 6872 containing ca. 5×10^4 colony forming units (cfu) were spotted onto seed medium agar plates containing the antibiotic and incubated at 27 °C. After 48 and 72 h, the plates were inspected for growth. The MIC is defined as the lowest concentration of antimicrobial agent which completely inhibited the visible growth.

2.4.5 Scanning electron microscopy (SEM)

Microphotographs of bacterial cells were taken according to Zellner *et al.* (1991), at 15 KV and the same magnification (x 10 000) with a Zeiss DSM 940 scanning electron microscope (Zeiss Oberkochen, Germany). Samples were taken, transferred immediately on membrane filters and fixed with Fix-buffer. After fixation, samples were dehydrated by gradually increasing the concentration of an ethanol bath and critical point drying (Balzers, Wiesbaden, Germany). The fixed samples were sputtered with gold / palladium (80:20%, w/w).

Inhibition of the cell-cycle of *C. ammoniagenes* using cephalexin, novobiocin, HU or MP was studied in seed medium. Cells were grown at 27°C to A_{660} = 0.1 (Kijima *et al.* 1998), then the inhibitor was added. Cells were incu-

bated with the studied inhibitor at the same temperature for 2 h. Afterwards, 1 ml was withdrawn and investigated with SEM. The control cultures were grown under the same conditios without addition of inhibitors.

Likewise, arrest of the cell-cycle of the ts-mutant CH31 was studied in seed medium. At the desired absorbance, the incubation temperature was shifted to the non-permissive temperature (37°C). After 2 h, samples were taken and examined with SEM. The morphological changes of the ts-mutant CH31 cells were also studied in the fermentation medium. For this purpose, sample was examined 24 h after shifting the incubation temperature to 37°C. Moreover the morphological changes of the IMP-productive cells grown in fermentation medium in 10-liter bioreactor were examined after 48 h. The control cultures of the ts-mutant were grown under the same conditions without shifting the incubation temperature.

Fix-buffer was prepared as follows:

DMA-buffer	52.8 ml
Glutaraldehyde 25%	7.2 ml

2.5 Molecular biological methods

Standard methods as described by Sambrook *et al.* (1989) were used unless otherwise indicated.

2.5.1 Determination of DNA concentration

The absorbance (*A*) of the DNA preparations was determined at 260 nm and 280 nm. $A_{260} = 1$ is equivalent to about 50 μg / ml double-stranded DNA. The quotient A_{260} / A_{280} gave the degree of DNA purity. The resulted value of pure DNA preparation should be between "1.8 - 2.0".

2.5.2 Agarose gel electrophoresis

The electrophoresis was run in 1X TAE buffer at 60-100 V. After electrophoresis, the gel was stained in 1X TAE containing 1% ethidium bromide for

15 min, destained in 1X TAE and then photographed under UV-light. The DNA molecular weight marker applied was lambda DNA-*Hind*III digest or 1-kb DNA ladders.

Buffers and solutions were prepared as follows:

Gel-loading buffer, 10X

Glycerol	50	%
EDTA/pH 8.0	50	mM
Bromophenol blue	0.15%	
Xylene cyanol	0.1	%

TAE-buffer, 1X

Tris-base	40	mM
Acetic acid	20	mM
EDTA, pH 8.4	1	mM

2.5.3 Digestion of DNA by restriction endonucleases

Digestion of DNA was done at the temperature and in the corresponding buffer as recommended by the supplier.

2.5.4 Isolation of DNA fragments

DNA fragments were extracted from agarose gels using a QIAEX II gel extraction kit according to the supplier.

2.5.5 Ligation

The plasmid vector was ligated with DNA fragments at ratio of 1:1 to 1:3 in a volume of maximal 20 μl. When cohesive-end fragments were involved, 1-3 U of T4-ligase was added and the mix was incubated at 16°C for 4 – 16 h. For ligation of the blunt-ended fragments about 50 times as much enzyme were added. When pXMJ19 or its derivatives were used the mix was incubated at 4°C for 16 h.

2.5.6 Isolation of chromosomal DNA

The chromosomal DNA of *Corynebacterium ammoniagenes* ATCC 6872 and ts-mutant CH31were extracted using the CTAB-method described by Ausubel *et al.* (1990). Five ml seed culture were inoculated with the bacterial strain and incubated at 27°C overnight. 2 ml of the overnight culture were centrifuged at 13000 rpm for 15 min. The pellet was resuspend in 567 µl TE-buffer containing lysozyme (10 mg / ml, Martin and Gil 1999) and incubated in a rotary shaker (180 rpm) at 37°C for 2 h. 30 µl of 10% SDS and 3 µl of 20 mg / ml proteinase K were added, mixed thoroughly and incubated 1 h at 37°C. To remove cell wall debris, denaturized protein, and polysaccharides complexed to CTAB, 100 µl of 5 M NaCl were added and mixed thoroughly. Afterwards, 80 µl of CTAB / NaCl solution were added, mixed, and incubated 10 min at 65°C. The CTAB-protein/polysaccharide complexes were extracted by using an approximately equal volume (0.7 to 0.8 ml) of chloroform / isoamyl alcohol (24:1). The mixture was mixed thoroughly and centrifuged at 13000 rpm for 4 to 5 min in microcentrifuge. The viscous supernatant was transferred to a fresh microcentrifuge tube, leaving the interface behind. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed thoroughly. The mix was centrifuged at 13000 rpm for 5 min. The supernatant was transferred to a fresh tube and the nucleic acids were precipitated by 0.6 vol. isopropanol. The tube was carefully inverted by hand until a stringy DNA precipitate became clearly visible. The chromosomal DNA was precipitated by spinning briefly at room temperature. The obtained DNA was washed with 70% ethanol to remove residual CTAB and respined 5 min at room temperature to re-pellet it. The supernatant was removed carefully and the pellet was briefly dried in a speed vacuum apparatus. The pellet was re-dissolved in 100 µl TE buffer.

Buffers and solutions were prepared as follows:

CTAB/NaCl solution
    CTAB   10 %
    NaCl   0.7 M

TE-buffer

Tris-HCl, pH 8.0	10	mM
EDTA, pH 8.0	0.1	mM

2.5.7 Mini-preparation of plasmid DNA

Plasmid DNA was extracted and purified according to Birnboim and Doly (1979). From the overnight culture, 2 ml were centrifuged at 13000 rpm for 15 min in microcentrifuge. The pellet was resuspended in 100 μl of solution A by vigorously vortex. 10 μl of 10 mg / ml RNase A were added and the mix was incubated at RT for 5 min. After that, 200 μl of freshly prepared solution B were added and mixed rapidly but gently. The mix was incubated on ice for another 5 min. Afterwards, 150 μl of ice-cold solution C were added, mixed gently, and incubated on ice for 5 min. To remove the precipitated proteins and chromosomal DNA, the mix was centrifuged at 13000 rpm for 5 min. For the purpose of sequencing, the DNA in the supernatant was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), and then once with chloroform: isoamyl alcohol (24:1). The plasmid DNA in the upper-phase was precipitated with 2 volumes of ethanol (-20°C) accompanied by 1/10 (v/v) 3 M sodium acetate at RT for 2 min. After centrifugation for 5 min, the pellet was washed with 70% ethanol (-20°C), dried and subsequently re-dissolved in 30-50 μl of TE buffer (or in 30-50 µl sterilized distilled water).
Buffers and solutions were prepared as follows:

Solution A

Glucose	50 mM
EDTA	10 mM
Tris-HCl	25 mM
pH	8.0

Solution B

SDS	1 %
NaOH	0.2 mM

Solution C

5 M CH$_3$ COOK	60	ml
acetic acid (glacial) 100%	11.5 ml	
Double distilled water	28.5 ml	
pH	4.8	

2.5.8 Midi-preparation of plasmid DNA

The method of mini-preparation of plasmid DNA was modified as follows to obtain high amounts of plasmid DNA with high purity. 50 ml LB-medium containing the selective antibiotic were inoculated with the desired *E. coli* strain and incubated overnight at 37°C. The cells were collected in a 50 ml polypropylene tube (Falcon-Tube 2070) by centrifugation at RT (5000 rpm for 10 min) in a Heraeus centrifuge (Cryofuge 5000). The pellet was resuspended in 5 ml solution A supplemented with 10 µg lysozyme and incubated at RT for10 min. After that, 10 ml solution B were added and mixed gently by inverting the tubes 5 to 6 times (don not vortex). After incubation on ice for 10 min, the cells should lysis and turn somewhat clear and viscous. 7.5 ml solution C was mixed gently, and the mixture was held on ice for 10 min. After centrifugation at 4°C (5000 rpm for 30 min) the supernatant (ca.20 ml) was transferred to a new tube and 0.6 vol. isopropanol (ca. 12 ml) was added. The mix was held at RT for 2 min and then was centrifuged at RT (5000 rpm for 10 min). The supernatant was excluded and the tube was placed in an inverted position on a paper for 5 min to allow the last traces of isopropanol to drain away. The pellet was resuspended in 2 ml autoclaved distilled water, then 2 ml 5 M LiCl-solution were added and the mix was incubated on ice for 15 min. The supernatant obtained after centrifugation at 4°C (5000 rpm for 15 min) was transferred to a new Falcon-Tube, 10 ml ice-cold ethanol (100%) were added and the mix was incubated overnight at -20°C. The mix was centrifuged at 4°C (5000 rpm for 30 min) and the pellet was resuspend in 400 µl TE buffer and transferred into a new Eppendorf-Cup. 5 µl RNase (10 mg / ml) were added and the mix was incubated at 37°C for 30 min. Phenol/chloroform/ isoamylalcohol extraction and DNA-precipitation were done as described previously (Sec. 2.6.8). The pellet was redissolved in 100-150 µl TE buffer (or in 150 µl sterilized distilled-water).

2.5.9 Polymerase chain reaction (PCR)

The DNA was *in vitro* amplified in a GeneAmp PCR system 9700 (Perkin Elmer Applied Biosystems, USA) according to Saiki *et al.* (1985). The mixture for a typical PCR is composed of:

Template DNA	x	µl
10x PCR buffer	5	µl
Mixture of four dNTPs, each at a concentration of 1.25 mM	10	µl
Primer 1 (10 µM)	1	µl
Primer 2 (10 µM)	1	µl
Taq DNA polymerase (5 U / µl)	0.25	µl
Double-distilled water to a final volume of	50	µl

The amount of template DNA applied is dependent on the concentration of target sequences to obtain 5 ng DNA in the final mix. The denaturation and DNA synthesis temperatures are generally 94°C and 72°C, respectively. The annealing temperature (T_{anneal}) is calculated by subtracting 4°C from the melting temperature (T_m) of the primer, which is calculated using the formula recommended by the following web site:

http://www.biotechlab.nwu.edu/OligoCalc.html

2.5.10 Southern blot

Southern blotting of DNA was performed according to the method described by Southern (1975) with some modifications.

2.5.10.1   DNA transfer from agarose gel to the nylon membrane

The stained DNA fragments were partially depurinized by soaking in depurinization solution for no longer than 10 min with gentle agitation, then denatured twice for 15 min each time in denaturation solution with gentle agitation. Afterwards, the gel was neutralized twice for 15 min each time in neutralization solution. DNA fragments were transferred to nylon membrane (Nytran[®] 0.45 µm, Schleicher & Schuell) with 20 x SSC by vacuum method using a Hy-

baid® vacuum blotting apparatus for 1 h. After transferring, the DNA was fixed to the membrane (120.000 μJ of UV energy) using autocross-link setting in a UV crosslinker, (UV Stratalinker 1800, Stratagene, La Jolla, CA, USA). The membrane was used immediately in the hybridization, or kept in the dark at RT for later application after drying.

2.5.10.2   *Preparation of digoxygenin-labeled probe by PCR*

The digoxigenin-labeled probes were prepared by the method described by Lanzillo (1990) in a PCR using a dNTP mix containing digoxigenin-11-dUTP (final concentration in PCR: dATP, dCTP, dGTP: 20 mM, dTTp: 0.15 mM, digoxigenin-11-dUTP: 0.05 mM). Primers CAE5 & CAE7 and OCA2-1 & OCA2-4 were used for preparation of probe E and probe F, respectively (Table 2.2).

2.5.10.3   *DNA-DNA hybridization with digoxigenin-labeled probe*

The DNA fixed on the nylon membrane was incubated with the pre-hybridization solution (20 ml per 100 cm^2 of membrane) in a hybridization oven (Biometra OV2, Biometra, Germany) for 2-4 h at 68 °C. The Dig-labeled probe was denatured by boiling at 100 °C for 10 min, and cooled immediately on ice. The hybridization solution was prepared by adding the denatured DNA probe to the pre-hybridization solution (5-25 ng / ml). The pre-hybridization solution was replaced with the hybridization solution (5 ml per 100 cm^2 of membrane) and incubated at the same temperature overnight. After hybridization the membrane was rinsed twice at 68°C for 5 min each time with the rinsing buffer I and the twice with rinsing buffer II for 15 min each time. Hybridization solution could be kept at -20°C for later use.

2.5.10.4 *Color detection with NBT/BCIP*

The membrane was rinsed in buffer 1 at room temperature for 5 min. In a heat sealed bag, the membrane was incubated with buffer 2 (20 ml per 100 cm^2 membrane) for 30-60 min with shaking. Buffer 2 was replaced with anti-Dig antibody solution (anti-digoxigenin-AP Fab fragments in buffer 2 at 1: 5000 at final concentration of 0.15 U / ml), and incubated for 30 min. The membrane was rinsed twice with buffer 1 for 15 min each time to remove the rest of anti-bodies solution. The membrane was equilibrated in buffer 3 for 2 min and finally incubated with developing solution (10 ml buffer 3 containing 45µl NBT solution and 35 µl BCIP solution) in the dark for 0.5-16 h. The developing reaction was stopped by rinsing the membrane in deionized water.

Buffers and solutions were prepared as follows:

BCIP solution
        5 mg / ml 5-bromo-4-chloro-3 indolyl-phosphate (X-phosphate) 4-toluidine salt in dimethylformamide (DMF).

Buffer 1

Maleic acid	0.1	M
NaCl	0.15	M
Tween 20	0.3	%
pH	7.5	

Buffer 2

Maleic acid	0.1	M
NaCl	0.15	M
Blocking reagent	1	%
pH	7.5	

Buffer 3

Tris-HCl	0.1	M
NaCl	0.1	M
MgCl$_2$	0.05	M
pH	9.5	

Depurinization solution

HCl	0.25	M

Denaturation solution
>   NaCl   1.5 M
>   NaOH   0.5 M

NBT solution
>   75 mg / ml nitro blue tetrazolium chloride in 70% (v/v) DMF

Neutralization solution
>   NaCl   2   M
>   Tris-HCl / pH 5.0   1   M

Pre-hybridization solution
>   5 x SSC
>   Sodium *N*- laurylsarcosinate   0.1 %   v/v
>   SDS   0.01%
>   Blocking reagent (Roche)   1   %

Rinsing buffer I
>   2 x SSC
>   SDS   0.1%

Rinzing buffer II
>   0.1 x SSC
>   SDS   0.1%

SSC-buffer, 20X
>   Tris-HCl, pH 7.5   0.3 M
>   NaCl   3   M
>   pH   7.5

TBS-buffer
>   Tris-HCl, pH 8.0   100 mM
>   NaCl   150 mM

2.5.11 DNA-Sequencing

Plasmid DNA and PCR fragments were analyzed by cycle sequencing, using the BigDye terminator cycle sequencing kit. The $nrdE^{ts}$ cloned in pUCECH31 or pUCEFCH31 and $nrdE^{+}$ cloned in pUCEF6872 were sequenced using the whole plasmids as DNA templates. These genes were sequenced in both directions (5′→3′ and 3′→ 5′) using specific sequencing primers (Table

2.2). The sequencing reactions were made on a GeneAmp®PCR system 9700. The reaction mix is composed of:

Template DNA	x µl
BigDye-Mix	2 µl
Specific primer (10 µM)	1 µl
HPLC water to a final volume of	10 µl

The amount of template DNA applied is dependent on the concentration of target sequences to obtain about 10 ng DNA in the final mix.

The PCR program was as follows:

	Temp. (°C)	Time	Cycle Nr.
Initial denaturation	96	2 min	1x
Denaturation	96	10 s	
Annealing	T_{anneal}	5 s	30x
Extention	60	4 min	
Cooling	4	∞	

The T_{anneal} was calculated according to the Tm of the specific primer used in each sequencing reaction (Sec. 2.6.9).

The PCR product was purified as recommended by the manufacturer and was sequenced on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystem Inc., Foster City, CA, USA).

2.5.12 Sequence analysis

The CLUSTAL W e-mail serves connected through the web site below were used for nucleotide and protein homology search as well as for multiple alignment analysis: (http://clustalw.genome.ad.jp/)

2.5.13   Methods of DNA-transfer

2.5.13.1   *Transformation of E. coli*

The plasmid vector with desired genetic element was introduced in the competent cells of *E. coli* strain as described by Sambrook *et al.* (1989).

2.5.13.2   *Electroporation of Corynebacterium ammoniagenes*

Transformation of plasmid vectors into *C. ammoniagenes* and ts-mutant CH31 was achieved according to the method described by Torrents *et al.* (2003) with some modifications. The strain was grown in 10 ml LB broth at 30°C until the culture reached an OD_{578} of 0.4–0.6. The cells were kept on ice for 5 min and harvested by centrifugation in a polypropylene tube at 5000 rpm. at 4°C for 10 min. The pellet was resuspended in 1 ml cold sterile distilled water and transferred into an Eppendorf tube. The cells were then washed three more times with distilled water in microcentrifuge. Any remaining supernatant was removed with sterile tips before resuspending the cells in 1 ml ice-cold 10% (v/v) glycerol. Finally, the cells were resuspended in 80 µl ice-cold 10% glycerol, and kept on ice prior to electroporation. For electroporation, 40 µl fresh electro-competent cells were mixed with plasmid DNA (1 µg) in a cold sterile electroporation cuvette (2 mm electrode gap, Biotechnologies and Experimental Research, BTX) and pulsed immediately with a BTX Electro Cell Manipulator ECM®600. The cell manipulator was usually set at a voltage of 2.5 kV. The cell solution was resuspended in 1 ml BHI (Oxoid) and then withdrawn immediately using a sterile Pasteur pipette and incubated at 37°C for 3 h before plating. In the case of electroporation of ts-mutant CH31, cell solution was incubated at 27°C for 3.5 h before plating on BHI agar plates containing chloramphenicol (30 µg / ml).

2.5.14 Amplification and sequencing of the *nrdE*$^+$ and *nrdE*^{ts} genes

The *nrdE*$^+$ (from *C. ammoniagenes* ATCC 6872) and *nrdE*^{ts} (from ts-mutant CH31) were amplified by PCR using XbCaE and SaCaE as forward and reverse primers, respectively (Table 2.2). For addition of a single adenosine to

the 3´-ends of the PCR products 7 µl from the PCR product was mixed with 1 µl dATP, 1 µl Taq-polymerase (1 unit / µl), and 1 µl Taq polymerase buffer (10 X). This mixture was incubated for 30 min at 72 °C. The PCR-products were ligated into vector pCR®2.1TOPO, which has single 3´-T overhangs. Ligation products were transferred into *E. coli* Top10 F´ and recombinant plasmids were identified by DNA sequencing (Sec. 2.5.11)

2.5.15   Genetic complementation of the ts-mutant CH31 with the *nrdE*⁺ from the wild-type ATCC 6872

The sequence of *nrdE*^{ts-} cloned in the plasmid pTopCH31 was identical with that of the ts-mutant CH31, without any failure. This gene was separated from the pTopCH31 plasmid by digestion with both XbaI and SacI, and then was ligated with the shuttle vector pXMJ19, linearized with the same restriction enzymes. The recombinant plasmid pXECH31 was introduced and amplified in *E. coli*. The other plasmid pXE6872 which contained the *nrdE*⁺ gene was constructed by substitution the sequence containing the point mutation with the corresponding sequence from the wild-type chromosomal DNA, which cloned previously in the pUCEF6872 (Sec. 3.2.4, Fig. 3.19). The obtained plasmids pXE6872 and pXECH31 were introduced, separately, and expressed in *E. coli* under induction conditions with 1 mM IPTG (Sec. 2.7.2.2). After successful expression of *nrdE*⁺ and *nrdE*^{ts} in *E. coli*, the pXE6872 and pXECH31 were introduced into the ts-mutant CH31 by electroporation (Sec. 2.6.13.2). Both plasmids were expressed in the ts-mutant CH31 at 27°C and observed by both Coomassie staining and immunoblotting using the specific antibody (rabbit polyclonal antiserum, Rb-anti-CEDAF, #2778).

2.5.16   Pre-induction of the expression of *nrdE*⁺ or *nrdE*^{ts} in the ts-mutant CH31

To investigate the biological activity of NrdE or NrdE^{ts} proteins in the ts-mutant background cells of *C. ammoniagenes* CH31 were transferred with plasmid pXE6872 or pXECH31. NBH-broth (50 ml) in conical flasks was inoculated with the desired strain and incubated overnight at 27°C. In test tube, 5

ml NBH broth was inoculate (10%) with the subculture and incubate for 3h at 27°C. Afterwards, 1 mM IPTG was added and incubated at 27°C for 0, 2, 4, or 6 h. The induced cultures were diluted and then plated on NBH-agar plates supplemented with 1 mM IPTG and chloramphenicol (30 µg / ml). The cell viability of the induced culture was determined (as cfu) at 27 and 37°C after 3 days.

2.6 Biochemical and biotechnological methods

2.6.1 Ribonucleotide reductase test

Mn-RNR activity with nucleotide-permeable cells was assayed according to Luo *et al.* (1997). For the 100 μl standard RNR assay, 1 x 10^8 cells were added to 6 mM DTT, 1 mM $MgCl_2$, and 0.25 μCi [^{3}H-CDP] (10–30 Ci / mmol) and 50 μM dATP as allosteric effector. After 20 min at 30 °C, the reaction was stopped by boiling for 3 min. Further treatment of the reaction mixture and automated nucleoside analysis and determination of radioactivity was carried out as described by Griepenburg *et al.* (1998). Blank values were obtained with heat-inactivated permeabilized cells (3 min boiling).

2.6.2 SDS-polyacrylamide gel electrophoresis (**SDS-PAGE**)

2.6.2.1   Preparation of gel

The mini-gel system (Biometra GmbH, Göttingen, Germany) with dimensions of 80 mm x 80 mm and thickness of 1 mm was employed in electrophoresis. The composition of resolving gel (12.5%) is given below (this amounts were sufficient for the mini-gel system)

30% acrylamide / 0.8% bis-acrylamide	3.15	ml
1.5 M Tris-HCl, pH 8.8	1.88	ml
30% glycerol	2.35	ml
10% SDS	75	µl
10% APS	75	µl
TEMED	5	µl

The polymerization is started after addition of the TEMED to the mix. The gel mold was filled with the resolving-gel solution up to about 2 cm below the plastic comb. The poured gel was immediately covered with double-distilled water. At the time of polymerization the stacking gel was prepared using the proportions given below:

30% acrylamide / 0.8% bis-acrylamide	1.25	ml
0.5 M Tris-HCl, pH 6.8	1.875	ml
10% SDS	75	µl
10% APS	75	µl
Double-distilled water	4.225	ml
TEMED	5	µl

The stacking gel solution was poured on the polymerized resolving gel and the comb was inserted fully to displace any air. After polymerization the gel could be used at once or packed with wet paper and sealed in a plastic bag and stored for about one week at 4°C. After loading the samples, 10 mA per gel were applied for 15 min then the current was increased at 20 mA per gel until the tracking dye reaches the bottom of the gel (about 90 min).

2.6.2.2   *Preparation of crude protein extract*

E. coli strain containing the desired plasmid was grown for overnight at 37°C in LBG medium in the presence of the suitable antibiotic. From this culture 2 x 2 ml were centrifuged and the pellet was washed in 400 µl LB. The pellet from one tube was resuspended in 5 ml LBG and incubated without induction. Pellet from the other tube was cultured in 5 ml LB medium and induced by addition of 1 mM IPTG for 3 h at 37°C. 2 ml from each culture were centrifuged and the pellets were resuspended in 200µl Laemmli-buffer and heated at 95°C for 5 min. The crude protein extract was loaded on SDS-Gels and further examined by Western blot analysis.

Induction with 1 mM IPTG of the ts-mutant CH31 containing the desired plasmid was done as described above by *E. coli*, but at 27°C. The cells from 2 ml induced culture were centrifuged at 13000 rpm for 8 min at 4°C. The pellet was washed in 1 ml 0.9% NaCl, and separated by centrifugation. The pellet was

incubated with 100μl lysis buffer at 37°C for 60 min. After that, 10μl 10% SDS were added. The sample was mixed with 100μl 2 x Laemmli buffer and heated at 95°C for 5 min. Buffers and solutions were prepared as follows:

Electrophoresis buffer
        Tris-HCl   25 mM
        Glycine   190 mM
        SDS   0.1   %
        pH   8.3

Laemmli buffer
        0.5 M Tris-HCl,pH6.8   25 ml
        10% SDS   40 ml
        Glycerol   20 ml
        2-mercaptoethanol   10 ml
        0.1% bromophenol blue   4 ml
        Double-distilled water   1 ml

Lysis buffer
        Tris-HCl,pH6.8   10 mM
        $MgCl_2$ 25 mM
        NaCl   200 mM
        Lysozyme (fresh)   5 mg / ml

2.6.3 Protein staining after SDS-PAGE (Coomassie stain)

After the electrophoresis, the gel was stained in the Coomassi staining solution for 30 min then destained until the background became pale blue. Coomassie solution was prepared as follows:

        Methanol   25 %
        Acetic acid   10 %
        Coomassie brilliant blue   0.1 %

2.6.4 Western blot

Western blot analysis was done according to the method described by Towbin *et al.* (1979). Two sheets of 3 MM filter papers were soaked in the transferring buffer and laid on the anode of a Semi-Dry Bloting Apparatus

(Biometra). The nitrocellulose membrane (Schleicher & Schuell) was placed on the stack of 3 MM filter paper. In all following steps, no air bubbles should be trapped between the gel, membrane, and filter paper. After the electrophoresis, the stacking gel was cut off and the resolving gel was placed on top of the nitrocellulose membrane, any trapped air bubbles should be squeezed with a gloved hand. Another two semidried sheets of the filter membrane were placed on top of the gel. The cathode was laid and the blotting was run at 0.8 mA / cm^2 for 1-1.5 h. After transferring, the membrane was incubated in blocking buffer (0.1 ml per 1 cm^2) in heat-sealed plastic bag at 37°C for 1 h in order to saturate free binding sites. The membrane was transferred to a new plastic bag and incubated overnight at 4°C (or at 37°C for 1 h) in the blocking buffer containing the first antibody (antibody raised against the protein of interest) diluted at 1:1000. The unbound antibody was removed by rinsing twice in buffer I for 30 min each time. To detect the specific binding of the first antibody, the membrane was incubated in the blocking buffer containing the second antibody (anti-rabbit IgG alkaline phosphatase conjugate) diluted at 1:2000 at 37°C for 1 h (Hawker *et al.* 1982). After rinsing twice in buffer I for 30 min each time, the membrane was equilibrated in buffer II for 5 min and then developed in 10 ml of developing solution generally for 10 min. The reaction was stopped in deionized water and the membrane was dried in the dark. Buffers and solution were prepared as follows.

Transferring buffer

Electrophoresis buffer	160 ml
Methanol	40 ml

Blocking buffer

Nonfat dried milk	5 g
Buffer I up to	100 ml

Buffer I

Tris-HCl	0.1	M
NaCl	0.1	M
MgCl$_2$	2.5	mM
Tween 20	0.05	%
pH	7.2	

Buffer II

Tris-HCl	0.1 M
NaCl	0.1 M
$MgCl_2$	25 mM
pH	9.5

Developing solution

    10 ml of buffer II containing 45 µl NBT solution and 35 µl BCIP solution)

2.6.5 Nucleotide fermentation

2.6.5.1 *NAD$^+$ production*

    Seed culture was prepared by inoculation of 100 ml seed medium (in 500ml conical flasks with baffles) with reactivated strain that was kept on slant agar at 4°C. The inoculated seed medium was incubated overnight at 27°C. Fermentation medium (45 ml in 500 ml conical flasks with baffles) was supplemented with DL-calcium pantothenate, Thiamine, Biotin, $MnCl_2$, and Urea. The supplemented fermentation medium was inoculated with the prepared seed culture to begin the fermentation process with $OD_{578} = 4$. After 7.5 h of fermentation at 27°C, growth of *C. ammoniagenes* was inhibited by addition of the studied inhibitor (2.4 µg / ml cephalexin, 24 µg / ml novobiocin, 20 mM HU, or 10 mM MP). Inhibition of growth was done by shifting the temperature to 37°C when the ts-mutant CH31 was used. As precursors for salvage nucleotide biosynthesis, adenine and nicotinic acid (2 mg / ml from each one) were added simultaneously with addition of the examined inhibitors or shifting the temperature.

2.6.5.2 *Assay of NAD$^+$*

    The concentration of NAD$^+$ was enzymatically determined by the method described by Ciotti and Kaplan 1957 and modified by Viereck 1975. Aliquots (1 ml) were withdrawn, the precipitated crystals were dissolved as described (Sec. 2.4.3.1). The reaction mixture contained 0.4 ml supernatant, 0.3 ml alcohol-

semicarbazide solution, 1.3 ml 0.1 M potassium phosphate buffer, pH 7.2. The reaction was started by drop-wise addition of a yeast alcohol dehydrogenase suspension (1 U / µl) and extinction was determined at 366 nm. The stable A_{366nm} − value obtained was designated as $E1$ and the blank value ($E0$) was determined without addition of ADH. The concentration of NAD^+ (mg / ml) was calculated by the following equation

$$\text{Concentration of } NAD^+ = 4.11 \, (E1 - E0).$$

Alcohol-semicarbazide solution was prepared as follows:

Semicarbazide-HCl	1.12 g
Ethanol	100 ml
Double-distilled water	100 ml

2.6.5.3   *IMP production in flasks*

The fermentation medium (45 ml in 500 ml conical flasks with baffles) was supplemented with DL-calcium pantothianate, Thiamine, Biotin, $MnCl_2$, and urea (only 3 g / l). The supplemented fermentation medium was inoculated with seed culture, prepared as mentioned above (Sec. 2.7.5.1) to begin the fermentation process with OD_{578} 4. Growth of the ts-mutant CH31 was inhibited after 7.5 h of fermentation at 27°C by shifting the incubation temperature to 37°C. Hypoxanthine (2.5 g / l), as the precursor for accumulation of IMP through the salvage pathway, was added to the fermentation broth just after shifting the temperature to 37°C. On the second day of fermentation, the residual amount of urea (3 g / l) was added and the pH of culture broth was adjusted with 25% NH_4OH between 7.5- 8.0 on the third day.

The IMP production conditions were optimized in flasks, yeast extract was replaced in the fermentation medium with corn steep liquor (CSL). Different amounts of CSL were used and IMP productivity was examined. In the further work 15 g / l CSL was optimized. Also, glucose feeding was modified in this study to improve the IMP productivity of the ts-mutant CH31. Forty percentage of glucose (52 g / l) was sterilized with the other components of the fermenta-

tion medium. The residual amount of glucose (60%) was sterilized separately and then added intermediately, 30% (39 g / l) on the second and third day of fermentation.

2.6.5.4   *Large scale production of IMP*

The ts-mutant CH31 was used for large scale production of IMP in a 10-liter bioreactor under the cultivation conditions which was optimized previously in flask-scale experiments. The strain was grown in seed medium at 27°C overnight. From this seed culture, mother culture was prepared by inoculation (10%) of flasks containing mixture of the seed medium and the fermentation medium (50:50 v / v). Only 50 g / l glucose was sterilized with the other components before starting the fermentation process. Vitamins and urea (3 g / l) were added just before inoculation with the mother culture. The 10-liter bioreactor (Biostat E, B-Braun Melsungen AG, Germany) containing 8 liters fermentation medium were inoculated (10%) with the mother culture and the cultivation conditions were adjusted as described in Fig 2.1
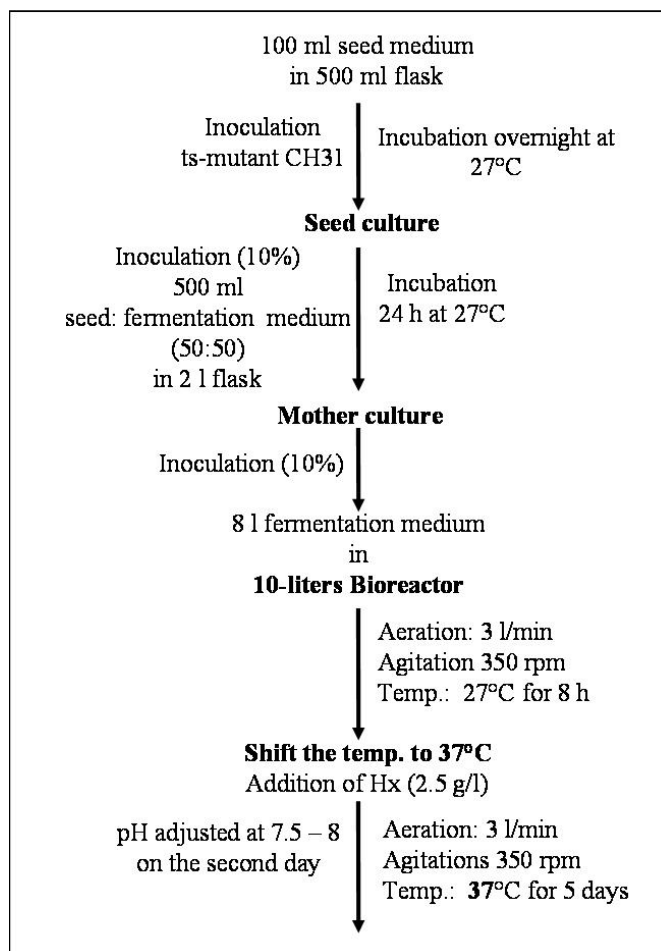
Fig. 2.1 Flow diagram of IMP production

The growth of the ts-mutant CH31 was followed by measured the OD_{578} and the dry cell weight was recalculated using a standard curve prepared previously (Sec. 2.4.3.2). Hypoxanthine (2.5 g / l) was added simultaneously with shifting the temperature to 37°C after 7.5 h. The residual amount of both glucose (40 g / l) and urea (3 g / l) were added 24 h after the onset of cultivation. On the second day, the pH was adjusted with 25% NH_4OH.

2.6.5.5   *Assay of IMP*

The accumulated inosine 5´monophosphate (5´-IMP) in the fermentation broth was assayed according to the method described by Wynants and van Belle (1985) and modified by Maessen *et al.* (1988). 1 ml culture broth was centrifuged at 14000 rpm for 10 min at 4 °C. The IMP was determined using HPLC system

(Knauer, Germany). The system consisted of a Knauer solvent organizer K-1500, Knauer variable wavelength monitor, and reverse phase column type Aqua 5 μ C18 125A (Phenomenex). Two solvent was used, solvent A and B. The solvent A was prepared by addition of 20 ml phosphoric acid to 800 ml distilled water. After adjustment of the pH of solvent A at 4 with 25% NaOH, the total volume was adjusted to 1 liter. The solvent B consisted of equal volumes of acetonitrile and methanol (50:50 v/v). Both solvents were filtrated through a 5 μm pore-size filter (Sartorius, Göttingen, Germany). 0.1 mM of the 5´-IMP (Sigma, $C_{10}H_{11}N_4O_8PNa_2$; 25.9% H_2O) was used as a standard. The analysis program was as follows:

- During the first 5 min the elution run was kept isocratic with 100% solvent A.
- Linear gradient started from 100% solvent A at 5 min to 85% solvent A and 15% solvent B at 15 min.
- During the next 5 min the mobile phase was restored to 100% solvent A.
- The new sample was introduced after 15 min re-equilibration with 100% solvent A.

The flow rate was kept constant at 0.7 ml / min.

3   RESULTS

3.1   Correlation between inhibition of cell-cycle of *C. ammoniagenes* and nucleotide production

3.1.1   NAD$^+$ production by inhibition of septum formation in *C. ammoniagenes* ATCC 6872

In order to investigate the metabolic correlation between the accumulation of the nucleotide (NAD$^+$) by *C. ammoniagenes* ATCC 6872 and the inhibition of its cell-cycle, cephalexin was used for inhibition of septum peptidoglycan synthetase. The **m**inimum **i**nhibitory **c**oncentration (MIC) of cephalexin against *C. ammoniagenes* ATCC 6872 was determined as 1.2 µg / ml when the efficacy of this antibiotic was examined in a complex medium (seed medium). The effect of cephalexin on cell morphology was studied during the exponential growth phase. Two hours after incubation in the presence of increasing concentrations of cephalexin (2, 4, 6, 8 or 10 x MIC) all cultures of *C. ammoniagenes* ATCC 6872 displayed lengthy cells (not shown) with characteristics of inhibition of cell division. Phase-contrast microscopy cannot answer the question whether septum formation was completely suppressed. The division-inhibited cells might have initiated septum formation but might not complete it. Therefore, the morphological changes induced by 2 x MIC were examined by scanning electron microscopy. The cells of the untreated control were able to complete their cell division (Fig. 3.1b) and short rods (0.5 - 1 µm) were observed as expected. A limited elongation was induced by addition of cephalexin and the culture displayed rods (1.5 - 2.5 µm) of twice the normal corynebacterial length. Moreover, many of the division-inhibited cells had initiated septum formation (see arrows in Fig. 3.1a).

(a)   **(b)**
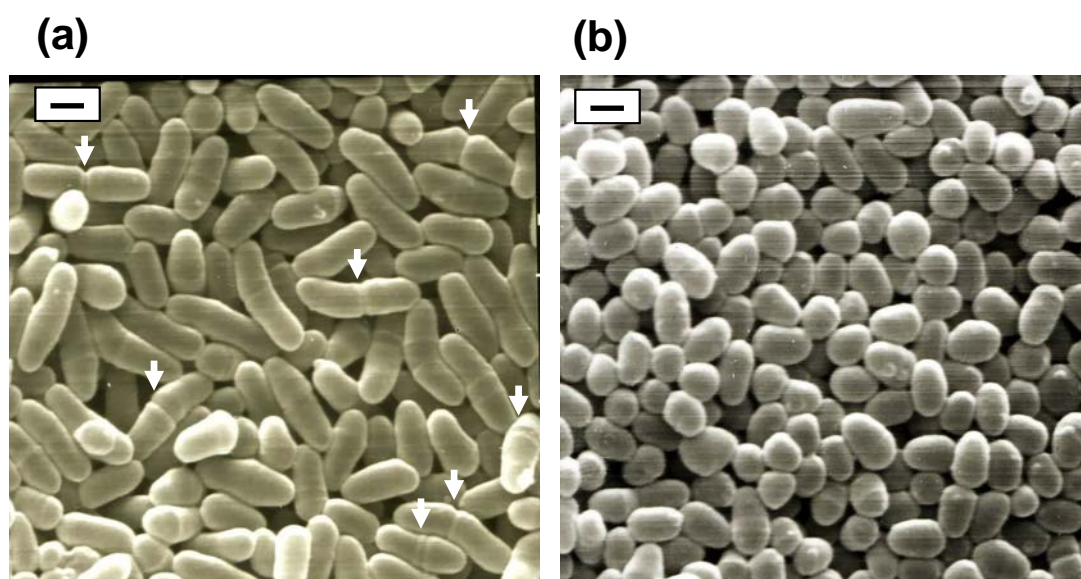
Fig. 3.1 Treatment (2 h) of *C. ammoniagenes* ATCC 6872 with 2 x MIC (2.4 µg / ml) cephalexin (a) versus control (b) as described in Materials & Methods (Sec. 2.4.5). The cells which have initiated but could not complete septum formation are indicated by white arrows. Scale bar = 1 µm.

To study whether such division-inhibited cells were NAD^+ producers, the manganese-requiring strain *C. ammoniagenes* ATCC 6872 was grown in synthetic fermentation medium supplemented with 10 µM $MnCl_2$. In this medium *C. ammoniagenes* showed balanced logarithmic growth for 10 h. Inhibition of cell division of *C. ammoniagenes* was again achieved by addition of 2.4 µg cephalexin per ml in the late phase of exponential growth (7.5 h). Nevertheless, increase of biomass continued even after inhibition of septum formation and the final growth yield was similar to the control (Fig. 3.2). However, the division-inhibited culture accumulated a threefold amount of NAD^+ compared to the untreated control. The maximum (0.48 g / l) of NAD^+ accumulation was detected five days after the onset of cultivation.
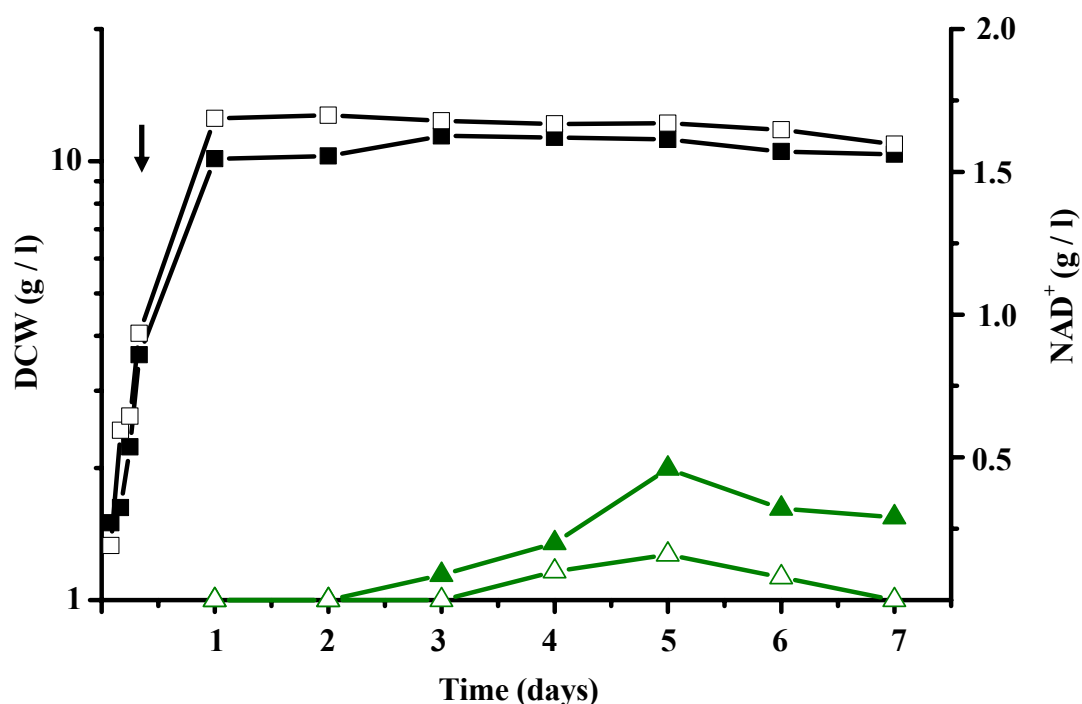
Fig. 3.2 Accumulation of NAD$^+$ (▲) due to perturbation with 2.4 µg cephalexin per ml during growth (■) of *C. ammoniagenes* ATCC 6872 in the manganese-supplied fermentation medium with pH adjustment (6.5-7.0). Cephalexin was added 7.5 h (arrow) after the onset of the cultivation. Open symbols refer to the unproductive, non-perturbated control. Growth was followed by measured OD$_{578}$ and recalculated as DCW from a standard curve previously prepared.

3.1.2 NAD$^+$ production by inhibition of DNA replication in *C. ammoniagenes* ATCC 6872

Novobiocin is known as an antibiotic that inhibits DNA replication by blocking the DNA-supercoiling catalyzed by DNA gyrase (Gellert *et al.* 1976; Maxwell 1999). MIC of novobiocin against *C. ammoniagenes* ATCC 6782 grown in seed medium was determined as 7 µg / ml. Increasing concentrations of novobiocin (2, 4, 6, 8, or 10x MIC) were added and their effects on cell mor-phology were investigated. Phase-contrast microscopy showed that novobiocin induced cessation of the cell-cycle in *C. ammoniagenes* at all levels applied

here (data not shown). Scanning electron micrography revealed that cells of *C. ammoniagenes* ATCC 6782 were elongated (rods of 1.5 – 2.5 μm) upon addition of 2x MIC of novobiocin and could not complete their cell division. Remarkably, they did not show any sign of septum formation (Fig.3.3a). Moreover, initiation of cell swelling was noticed due to inhibition of cell-cycle.
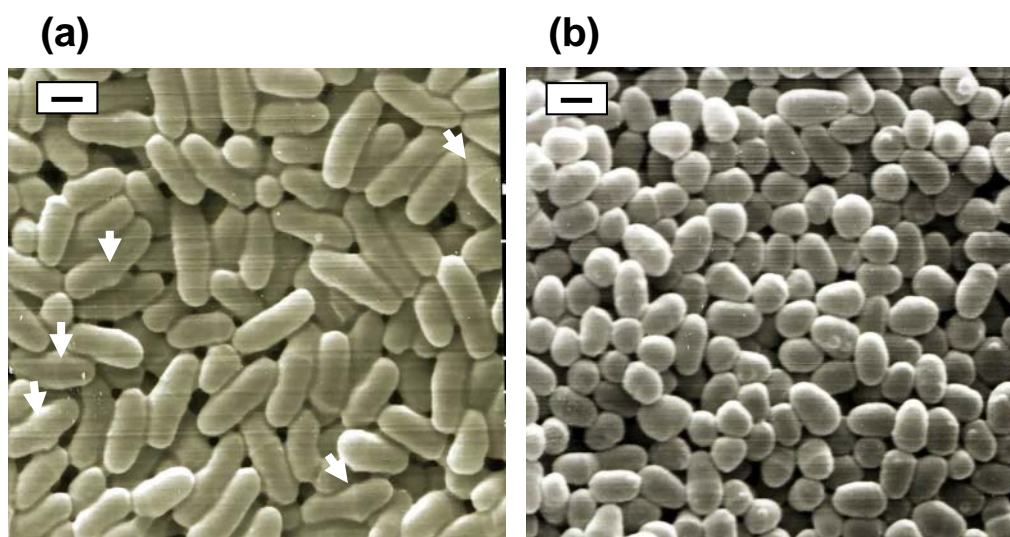
Fig. 3.3 Treatment (2 h) of *C. ammoniagenes* ATCC 6872 with 2 x MIC (14 μg / ml) novobiocin (a) versus control (b) as described in Materials & Methods (Sec. 2.4.5). Induction of cell swelling was indicated by white arrows. Scale bar = 1 μm.

The ability of *C. ammoniagenes* ATCC 6872 to accumulate NAD^+ was examined, when the cell-cycle was inhibited using the antibiotic novobiocin. For this purpose, exponential growth of *C. ammoniagenes* in Mn^{2+}-supplemented fermentation medium was blocked by addition of 2x MIC of novobiocin 7.5 h after the onset of the cultivation. Irrespective of inhibition of cell-cycle by novobiocin a continued increase of biomass was observed as in the case of arrest of cell-cycle using cephalexin. Detectable amounts of NAD^+ were obtained 48 h after the onset of cultivation process (Fig. 3.4). Also, the maximum of NAD^+ accumulation (0.56 g / l) was observed in the culture broth after five days.
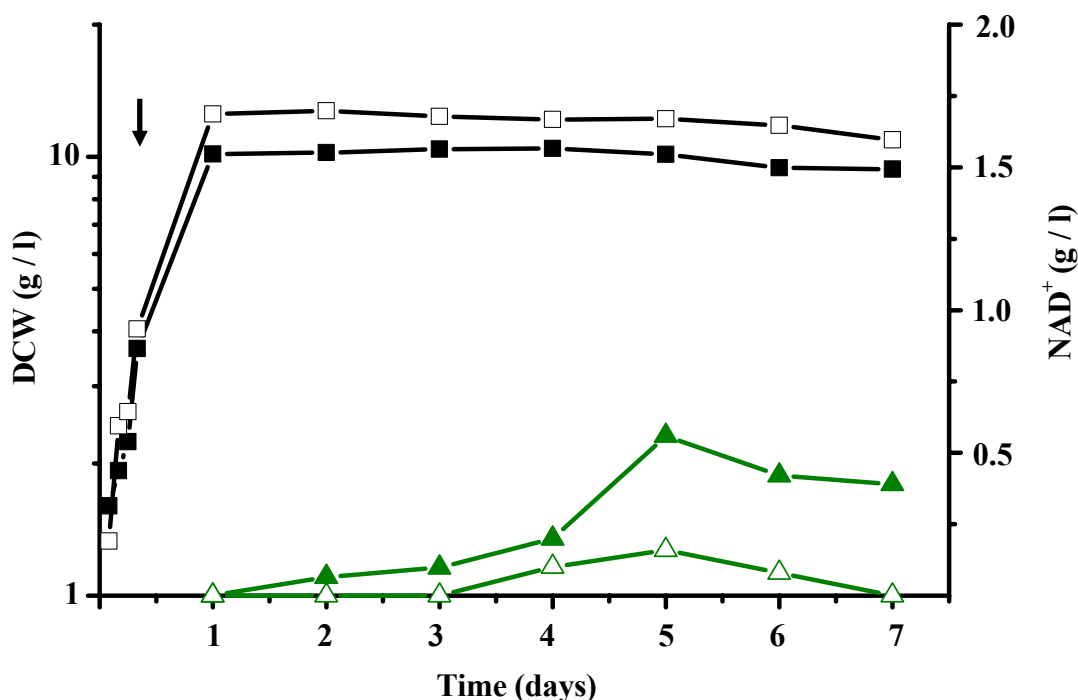
Fig. 3.4 Accumulation of NAD⁺ (▲) due to perturbation with 14 µg novobiocin per ml during growth (■) of *C. ammoniagenes* ATCC 6872 in the manganese-supplied fermentation medium with pH adjustment (6.5-7.0). Novobiocin was added 7.5 h (arrow) after the onset of the cultivation. Open symbols refer to the unproductive, non-perturbated control. DCW was calculated as described in Fig. 3.2.

3.1.3 NAD⁺ production by inhibition of DNA precursor biosynthesis

3.1.3.1   Inactivation of Mn-RNR in C. ammoniagenes ATCC 6872 by addition of radical scavengers

The stable free-radical of the Mn-RNR of *C. ammoniagene*s (Griepenburg *et al.* 1996 & 1998) is sensitive to the radical scavengers hydroxyurea (HU) and *p*-methoxyphenol (MP). Here, the proper inhibition of DNA precursor biosynthesis in *C. ammoniagenes* was studied by adding these radical scavengers at 20 mM (HU) or 10 mM (MP) to exponential cultures growing in seed medium.

Cultures treated with HU or MP had elongated rods of 1.5 – 2.5 µm length, whereas the control culture had normal short-rods about 0.5 – 1 µm, (Fig. 3.5) as expected. This result indicates that inhibition of Mn-RNR did not allow the cells to complete division, noticeable by formation of rods with only limited elongation. This led to an inevitable arrest of cell-cycle of *C. ammoniagenes* (Fig. 3.5). Remarkably, elongated cells of *C. ammoniagenes* did not display any sign of initiated septum formation upon arrest of the cell-cycle with HU.

(a)

(b)

(c)
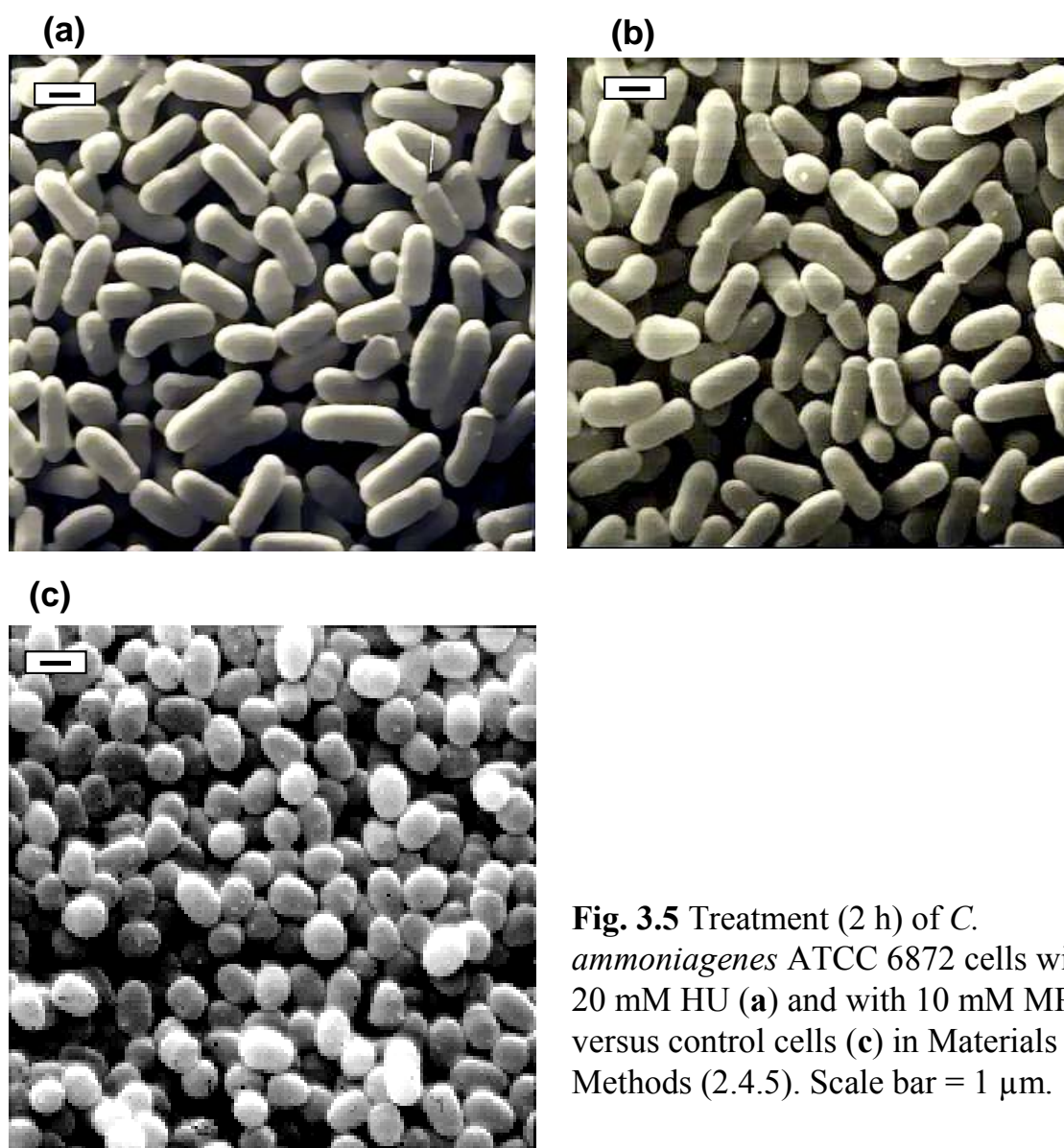
Fig. 3.5 Treatment (2 h) of *C. ammoniagenes* ATCC 6872 cells with 20 mM HU (**a**) and with 10 mM MP (**b**) versus control cells (**c**) in Materials & Methods (2.4.5). Scale bar = 1 µm.

The ability of *C. ammoniagenes* ATCC 6872 to produce NAD^+ upon deliberate cell-cycle arrest was investigated in the Mn^{2+}-supplemented fermentation medium by inhibition of Mn-RNR. The radical scavengers HU (20 mM) or PM (10 mM) were added to this medium 7.5 h after the onset of cultivation. Remarkably, treatment with both radical scavengers led to an increase of biomass similar to the untreated control. The extracellular accumulation of NAD^+ of the untreated culture never exceeded background values of 0.16 gram per liter (Fig. 3.6 a and b). Perturbation of growth with 20 mM HU led to increased (sixfold) level of NAD^+ compared to the control (Fig. 3.6a). After five days of cultivation in the presence of HU, 1.38 g NAD^+ per liter was accumulated in the cultivation broth. Upon addition of 10 mM MP accumulation of NAD^+ was enhanced fourfold. The maximum (0.8 g / l) was obtained after three days of cultivation (Fig. 3.6b).

To confirm that inhibition of DNA precursor biosynthesis generates the overproduction of NAD^+, it was necessary to assay the level of ribonucleotide reduction with and without perturbation by HU or MP. For this purpose, the activity of Mn-RNR was determined periodically withdrawn before and after addition of radical scavengers (Fig. 3.7). When the balanced growth was not perturbated by any addition the maximum of RNR activity was observed in the late exponential phase. The steep increase towards and the sharp decline after the maximum of RNR activity, characteristic of a "peak" enzyme, is a well-known behavior of the Mn-RNR of *C. ammoniagenes* (Auling and Follmann 1994). However, upon addition of 20 mM HU after 7.5 h a premature (Fig. 3.7a) decline of RNR activity was measured. Similar results were obtained by addition of 10 mM MP (Fig. 3.7b).
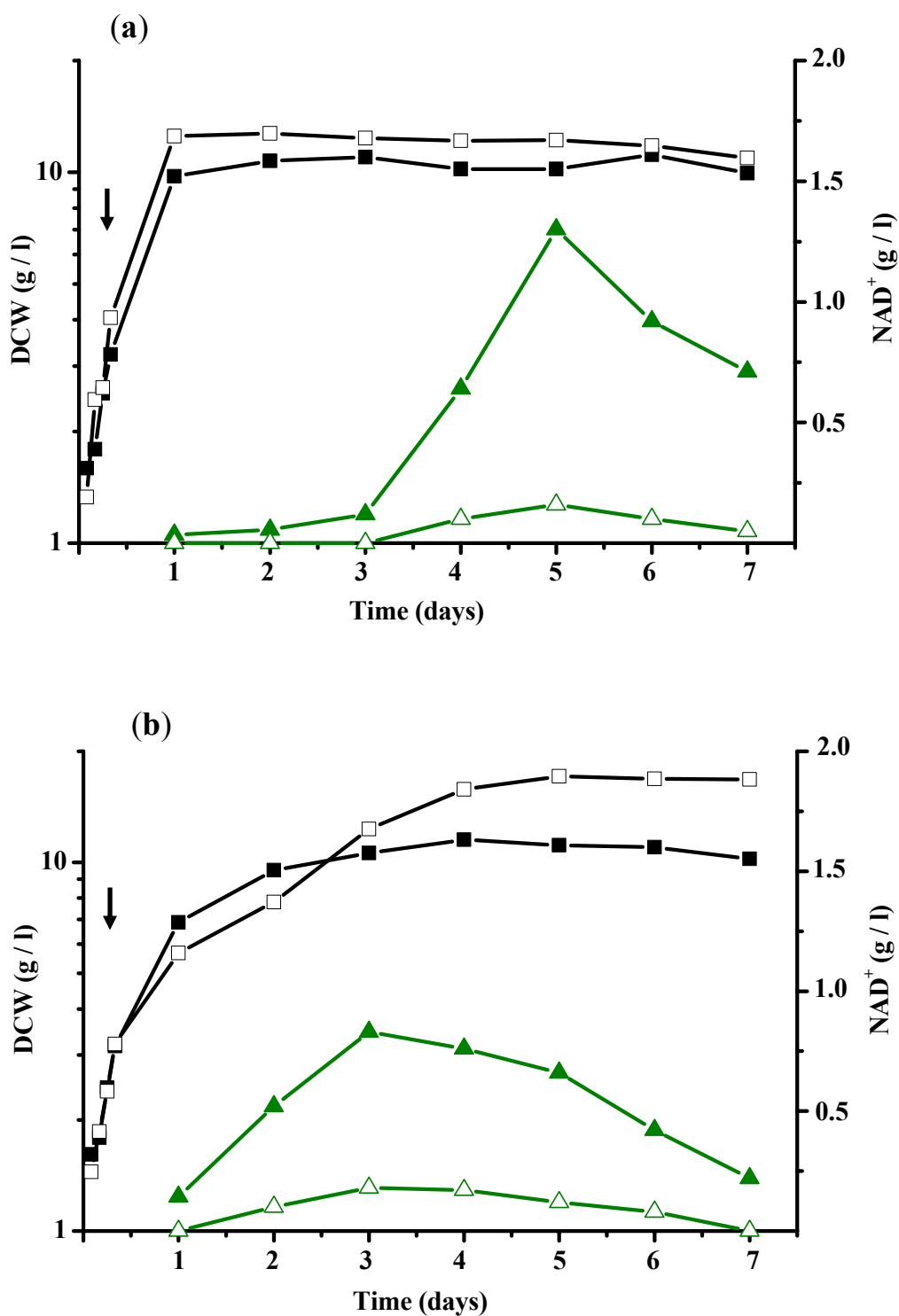
Fig. 3.6 Accumulation of NAD⁺ (▲) due to perturbation with 20 mM HU during growth (■) of *C. ammoniagenes* ATCC 6872 in the manganese-supplied fermentation medium with pH adjustment (6.5-7.0). Hydroxyurea (a) or *p*-methoxyphenol (b) was added 7.5 h (arrows) after the onset of the cultivation. Open symbols refer to the unproductive, non-perturbated control. DCW was calculated as described in Fig. 3.2.
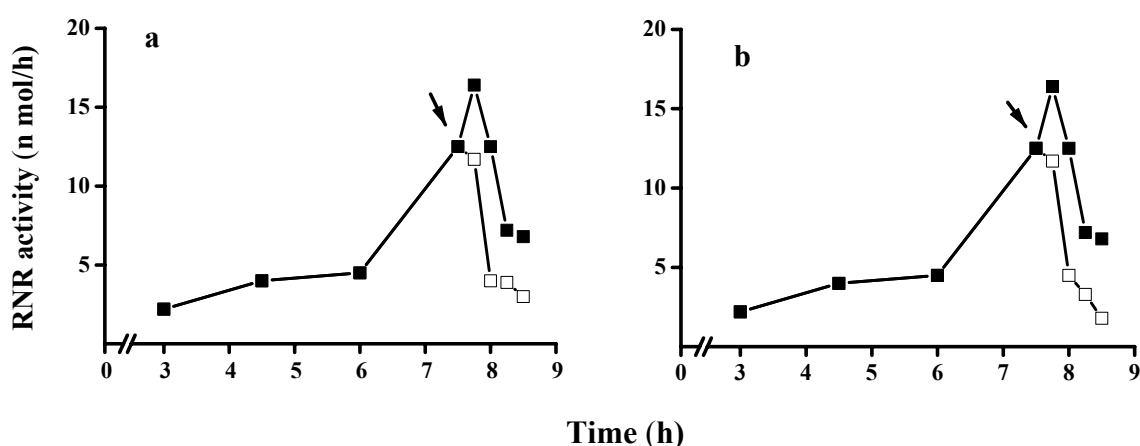
Fig. 3.7 Regulated shut-down ('peak' behavior) of ribonucleotide reduction (■) in *C. ammoniagenes* ATCC 6872 versus deliberate inactivation (arrow) of RNR-activity due to radical scavenging (open symbols) with 20 mM HU (a) and 10 mM MP (b)[*]. RNR activity was assayed using nucleotide-permeable cells as described in section 2.6.1.

3.1.3.2 Inhibition of ribonucleotide reduction in the ts-mutant CH31 by temperature shift

Induction of unbalanced growth by inhibition of the Mn-RNR in *C. ammoniagenes* ATCC 6872 wild-type using radical scavengers was shown in the previous section. Changing the cultivation temperature of the thermosensitive mutant CH31 to a certain level above 30 °C results in inhibition of the ribonucleotide reductase. Initially, 40 °C was chosen as a non-permissive temperature (Luo *et al.* 1997). In the present study 37°C was chosen as a non-permissive temperature. The inhibition of cell-cycle of the ts-mutant CH31, grown in seed medium, was observed by shifting the temperature from 27°C to 37°C (Fig. 3.8). Under these non-permissive conditions a limited elongation (twice of the normal cells) was induced. At 27°C, the ts-mutant CH31 showed normal rods (0.5- 1.5).

---

[*] The RNR activity with radio labeled substrate was determined with the aid of Dr. Abbouni as described in Materials & Methods (Sec. 2.6.1).
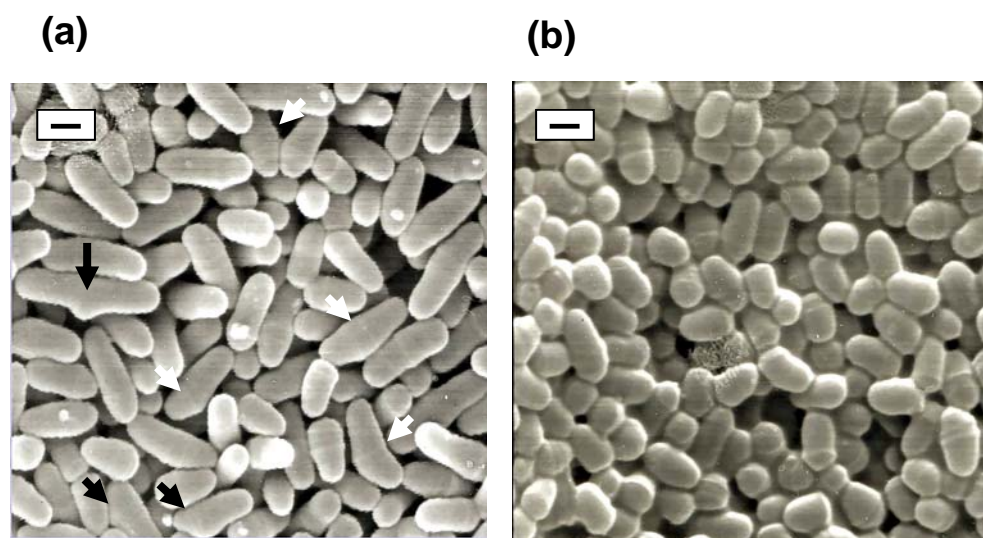
Fig. 3.8 Induction of limited elongation by ts-mutant CH31cells (a) due to shifting the temperature to 37°C versus control culture (b) grown at 27°C as described in Materials & Methods (Sec. 2.4.5). Induction of cell swelling was indicated by white arrows, whereas branched (T-shaped) cells were indicated by black arrows. Scale bar = 1 µm.

Biochemically, the ts-mutant CH31 has a putative genetic defect in the *nrdE* gene encoding the large subunit (NrdE, also known as R1E) of the Mn-RNR (Luo *et al.* 1997). The suitable time for inhibition of Mn-RNR to induce unbalanced growth in fermentation medium was 7.5 h (see Fig. 3.7). Therefore, induction of unbalanced growth by shifting the temperature to 37°C at this time was investigated for accumulation of NAD^+. The continued increase of biomass even after the temperature shift (Fig. 3.9) indicated unbalanced growth of the ts-mutant CH31. Under this condition the ts-mutant CH31 exhibited a remarkable increase in the NAD^+ productivity (Fig. 3.9). The accumulation of NAD^+ started two days after the temperature shift. The highest amount of NAD^+ (1.52 g / l) was accumulated after four days. At 27°C the NAD production of the ts-mutant CH31 (Fig. 3.9) was inefficient as observed when cultivating the wild-type strain *C. ammoniagenes* ATCC 6872 at this temperature (Fig.3.6).
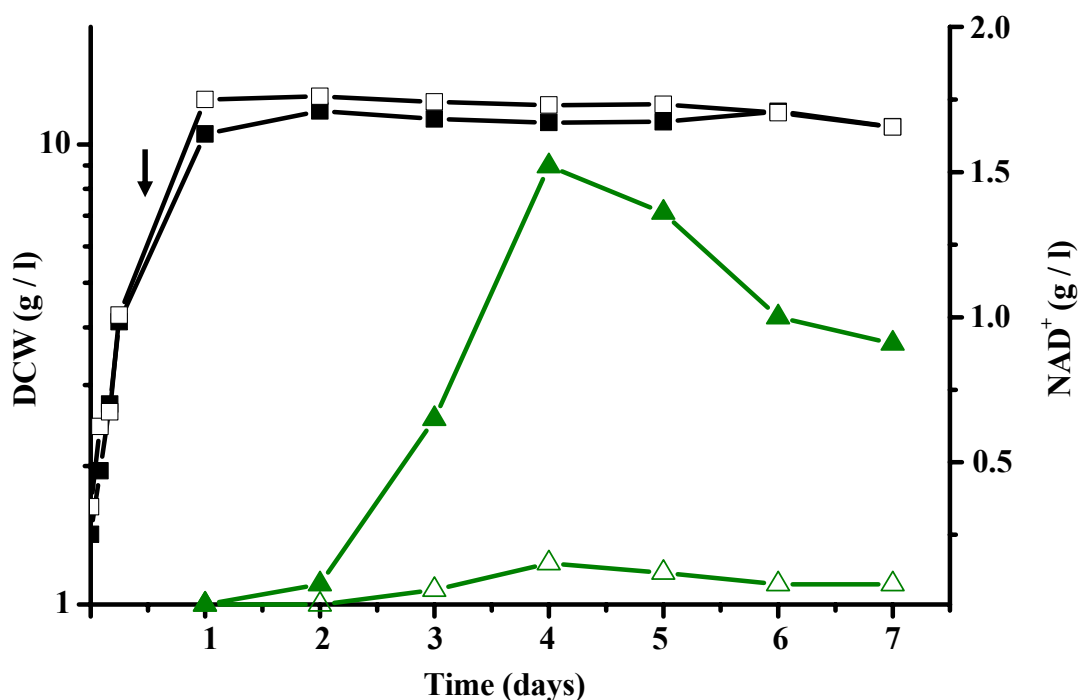
Fig. 3.9 Accumulation of NAD⁺ (▲) due to perturbation by temperature shifting to 37°C during growth (■) of the ts-mutant CH31 in the manganese-supplied fermentation medium with pH adjustment (6.5-7.0). Temperature was shifted 7.5 h (arrow) after the onset of the cultivation. Open symbols refer to the unproductive, non-perturbated control grown at 27°C. DCW was calculated as described in Fig. 3.2.

3.2 Identification of the putative point mutation in the ts-mutant CH31 and correlation with its thermosensitive phenotype

After studying the fundamentals of the correlation between arrest of cell-cycle and nucleotide production (Sec. 3.1), it was clearly stated that the highest amount of NAD⁺ was accumulated when the cell-cycle of the ts-mutant CH31was inhibited by temperature shift. However, prior to use this mutant strain in the production of IMP as a commercial flavor enhancer, it was necessary to identify the putative point mutation in its *nrdE* gene which may be responsible for its thermosensitive phenotype.

3.2.1 Cloning and sequencing of the *nrdE*[ts] gene of strain CH31

In order to identify the putative point mutation of the *nrdE*[ts] gene of strain CH31 a 2 kb *Bam*HI-*Sph*I fragment of genomic DNA of CH31 was cloned in pUC18. The resulting plasmid pUCECH31 was introduced and *in vivo* amplified in *E. coli* XL1-Blue. The primers CAE1 – CAE12 (Fig. 3.15) allowed to sequence nearly the complete (2157 of 2163 bases) gene. Since the sequence of *nrdE* gene from the parent strain ATCC 6872 (Oehlmann 1998) available in our laboratory is not complete, the sequence of *nrdE*[ts] (Fig. 3.16) was compared with *nrdE* sequence of the same strain (ATCC 6872) published in the GenBank[®] [gi:3077610] by Fieschi *et al.* (1998). Upon sequence alignment of both genes four base pair substitutions were detected in the *nrdE*[ts] gene.The first base pair substitution was at the nt 1301, and the other three changes were near to the C-terminus, at nt 2021, 2032, and 2063 (Fig. 3.10).

```
CA-BG   TGCAACCTCGGCTCCATGAACATTGCTTTGGCGATGGATTCTCCAGACTTCGGTGGCACG   1320
CH31    TGCAACCTCGGCTCCATGAACATTGCTTTGGCGATGGATTTTCCAGACTTCGGTGGCACG   1320


CA-GB   AAGTACGTCGACCAGGGCTTGTCTTTGACCTTGTTCTTCAGGGACACCGCGGCCACCCGC   2040
CH31    AAGTACGTCGACCAGGGCTTGTCTTTGACCTTGTTCTTCAAGGACACCGCGACCACCCGC   2040

CA-GB   GACATCAACCGCGCGCAGATCTTCGCATGGCGCAAGGGCATTAAGACCTTGTACTACATC   2100
CH31    GACATCAACCGCGCGCAGATCTACGCATGGCGCAAGGGCATTAAGACCTTGTACTACATC   2100

CA-GB   CGCTTGCGTCAGATGGCGCTGGCTGGAACCGAGATTGAAGGTTGCGTCTCCTGCATGCTCTAA   2163
CH31    CGCTTGCGTCAGATGGCGCTGGCTGGAACCGAGATTGAAGGTTGCGTCTCCTGCATG         2163
```

Fig. 3.10 Nucleotide sequence alignment of the *nrdE*[ts] from the ts-mutant CH31 and the wild-type ATCC 6872 (CA-GB) available in GenBank[®] [gi:3077610]. The base pair substitutions are highlighted.

3.2.2 Simultaneous cloning of Mn-RNR genes (*nrdEF*) of *C. ammoniagenes* ATCC 6872 and the ts-mutant CH31

The finding of three base-pair substitutions concentrated in the C-terminal region was rather unexpected. The mutant CH31 was isolated (Luo *et al.* 1997) by random mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanosine (MNNG) which generates mainly point mutations To solve this inconsistency it appeared necessary to compare the *nrdE* genes of CH31 and its parent strain maintained in the institute´s culture collection. The new strategy was to clone a 5.2 kb *Xma*I fragment of the *nrd*-operon (Fig. 3.11) containing the *nrdE* and *nrdF* genes from both strains (CH31 and its parent strain ATCC 6872) simultaneously. In order to obtain these full-length clones specific-probes for each of the *nrdEF* genes were generated and applied in the gene cloning protocol as depicted in Fig. 3.11.
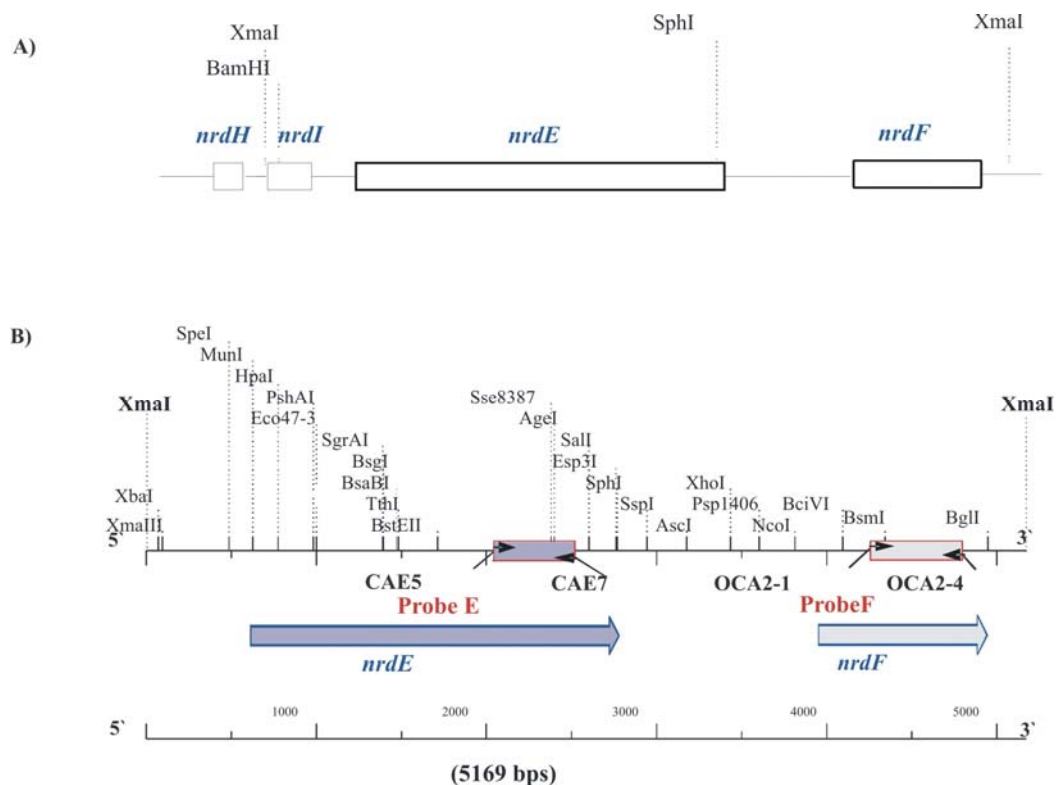
Fig. 3.11 Genetic map of the *nrd*-operon (A) and restriction maps and *nrdE* and *nrdE* genes located on the 5.169 kb *Xma*I-fragment (B) used for sequencing of *nrdE* from ts-mutant CH31 and the parent strain ATCC 6872. The solid black arrows indicate the location and direction of cloning primers (CAE5/CAE7) and (OCA2-1/OCA2-4) used for generation of dig-labeled probe E and F, respectively.

Four oligonucleotide primers were synthesized (Table 2.2) on the base of the known sequences of *nrdE* and *nrdF* from the wild-type strain *C. ammoniagenes* ATTC 6872 (Fieschi *et al.* 1998; Oehlmann *et al.* 1998). Probe E (380 bp) was amplified from the chromosomal DNA of the wild-type strain ATCC 6872 using primers CAE5 and CAE7. Likewise probe F was produced using primers OCA2-1 and OCA2-4. Both probes were labeled with digoxigenin-11-dUTP during their amplification. For cloning of the 5.2 kb fragment, the chromosomal DNA of both strains were digested with *Xma*I and then separated by electrophoresis. DNA fragments containing the *nrdEF* genes were identified by southern blot using probe E or probe F. (Fig. 3. 12).
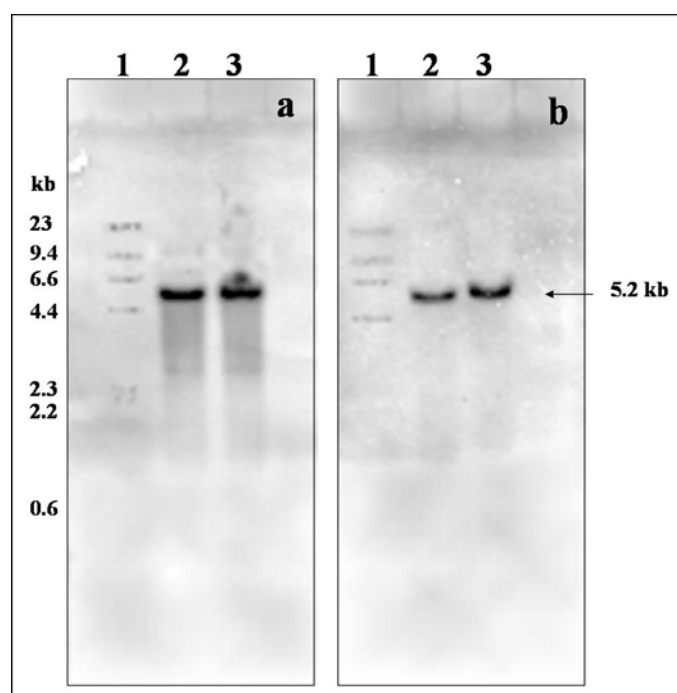
Fig. 3.12 Southern blot analysis of chromosomal DNA of *C. ammoniagenes* ATCC 6872 and ts-mutant CH31 using probe E (a) and probe F (b). Lane 1: Lambda DNA-*Hin*dIII digest, digoxigenin-labeled, as a molecular weight marker; lane 2: Chromosomal DNA of *C. ammoniagenes* ATCC 6872, digested with the *Xma*I; lane 3: Chromosomal DNA of ts-mutant CH31, digested with the *Xma*I.

DNA fragments from the region 4.7 - 5.5 kb (Fig 3.13) were excised from the agarose gel, subdivided into four fractions, and extracted using a QIAEX II gel extraction kit. The isolated fragment pools which hybridized with both probes were ligated into the *Xma*I-site of pUC18.
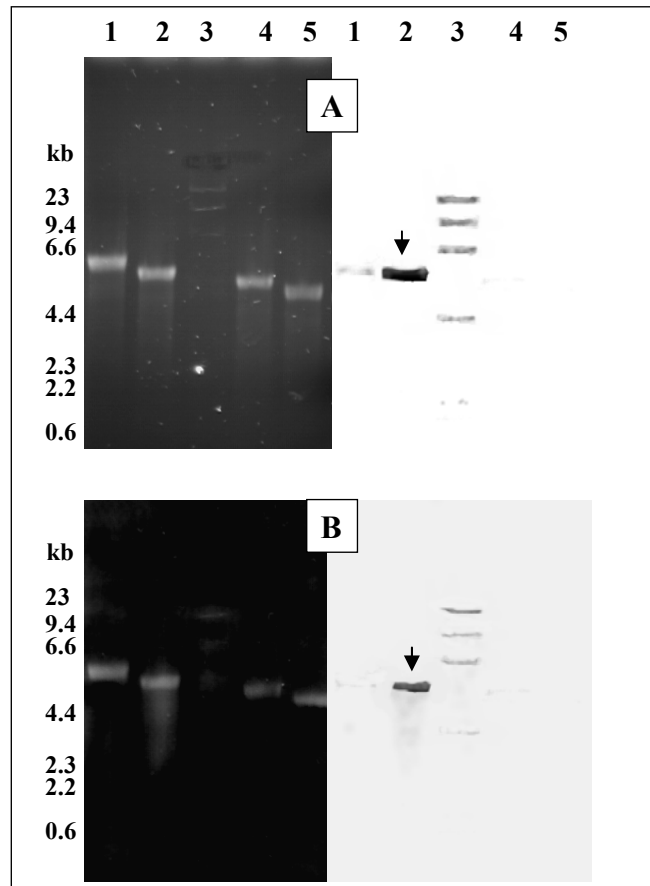
Fig. 3.13 Electrophoresis of genomic DNA of *C. ammoniagenes* ATCC 6872 (**a**) and the *nrdE*^{ts} mutant CH31 (**b**), in the range of 4.7 to 5.5 kb, which were subdivided into four fractions: lane 1: 5.5-5.3 kb, lane 2: 5.3-5.1 kb, lane 4: 5.1-4.9 kb, lane 5: 4.9-4.7. The 5.2 kb DNA fragments contained the *nrdE* genes were detected by southern blotting with Probe E and indicated with arrows. Lambda DNA-*Hin*dIII digest, digoxigenin-labeled, as a molecular weight marker (Lane 3)

For *in vivo* amplification the recombinant plasmids were transformed into competent cells of *E. coli* XL1-Blue and submitted to blue-white screening. A total of 150 plasmid mini-lysates, extracted from the white colonies (75 for wild-type strain and 75 for ts-mutant CH31), were digested with *Xma*I. By

Southern blotting with probe E or probe F only one from each group of the re-combinant plasmids hybridized with both specific-probes (Fig.3.14) and thus con-tained the desired insert. The resulting plasmids were designated pUCEF6872 for insert DNA of the wild-type strain *C. ammoniagenes* ATCC 6872 and pUCEFCH31 when containing the 5.2 kb *Xma*I-DNA fragment isolated from the ts-mutant CH31.
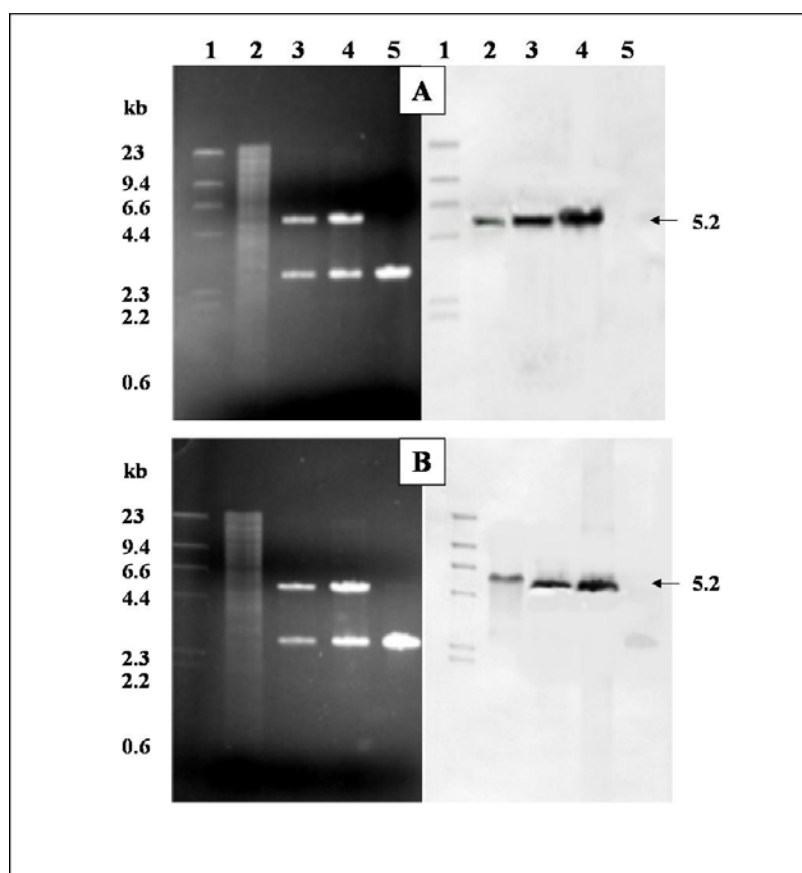
Fig. 3.14 Electrophoresis and southern blot detection of recombinant plasmids harboring a 5.2 kb *Xma*I-fragment from *C. ammoniagenes* ATCC 6872 and ts-mutant CH31 using probe E (A) and probe F (B).
Lane 1: lambda DNA-*Hin*dIII digest, digoxigenin-labeled, as a molecular weight marker; lane 2: chromosomal DNA from *C. ammoniagenes* ATCC 6872, digested with *Xma*I, as appositive control; lane 3: recombinant plasmid containing a 5.2 kb *Xma*I-fragment from *C. ammoniagenes* ATCC 6872, digested with *Xma*I; lane 4: recombinant plasmid contain-ing a 5.2 kb *Xma*I-fragment from ts-mutant CH31, digested with *Xma*I; lane 5: pUC18 digested with *Xma*I, as a negative control.

3.2.3   Sequence comparison between *nrdE⁺* and *nrdE^{ts}*

The plasmids pUCEF6872 and pUCEFCH31 were *in vivo* amplified in *E. coli* XL1-Blue. Plasmid DNA was extracted and purified as described in Materials & Methods. The *nrdE* from the wild-type and CH31 strains were sequenced using specific-oligonucleotide primers (Table 2.2). Sequencing strategy, primers and sequencing direction, for *nrdE* gene is shown in Fig. 3.15.

(5169 bps)
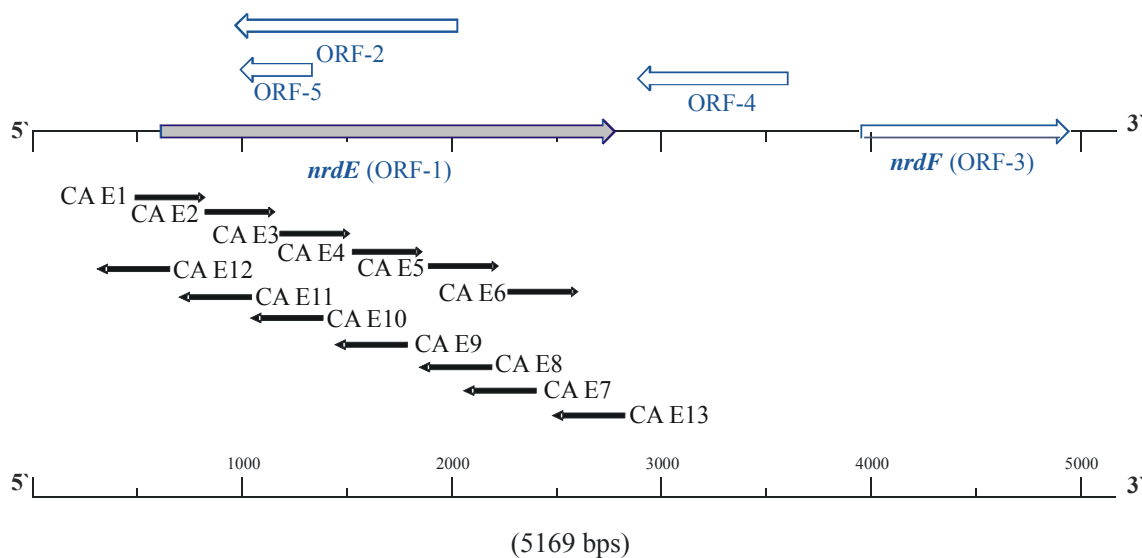
Fig. 3.15 Open reading frames found in *Xma*I-DNA fragment from *C. ammoniagenes* and sequencing strategy for *nrdE* gene cloned in both pUCEF6872. The solid-black arrows indicate the position and direction of sequencing length within the *nrdE* gene. The primers used for sequencing were also noted. The known sequence of 5.169 kb *Xma*I-DNA fragment the *nrdE* gene was defined as an open reading frame (ORF) of 2163 bp with GTG as the initiation codon at nt 616 and TAA at nt 2778 as a stop codon. The *nrdF* ORF is 992 bp in length from nt 3951 to 4940 (Fieschi *et al.* 1998).

By comparison between the obtained sequences of *nrdE* from both ts-mutant CH31 and its parent strain *C. ammoniagenes* ATCC 6872, it can be clearly concluded that the *nrdE* gene sequence of ts-mutant CH31 was identical to that of the parent strain ATCC 6872, except at nt 1301. At this position the nucleotide is cytosine in the wild-type strain and thymine in the *nrdE* gene of ts-mutant CH31 (Fig. 3.16).

```
CA      GTGACTCAACAATTGGGCAAAACCGTTGCCGAGCCGGTAAAGAATTCCGAGAAGCTAGAC      60
CH31    GTGACTCAACAATTGGGCAAAACCGTTGCCGAGCCGGTAAAGAATTCCGAGAAGCTAGAC      60

CA      TTCCATGCTCTCAACGCACTGTTGAACCTGTATGACGAAGACGGCAAAATTCAGTTCGAT     120
CH31    TTCCATGCTCTCAACGCACTGTTGAACCTGTATGACGAAGACGGCAAAATTCAGTTCGAT     120

CA      AAAGACCGTGAAGCTGCCAACCAGTACTTCTTACAGCACGTTAACCAAAACACGGTCTAC     180
CH31    AAAGACCGTGAAGCTGCCAACCAGTACTTCTTACAGCACGTTAACCAAAACACGGTCTAC     180

CA      TTCCACGACCTGGAAGAAAAGATTGAATACTTAGTTGAAAACAAGTACTACGAGCCAGAA     240
CH31    TTCCACGACCTGGAAGAAAAGATTGAATACTTAGTTGAAAACAAGTACTACGAGCCAGAA     240

CA      GTTATCGAAGCTTATGACTGGGAATTCATTAAGTCACTGTTTAAGCGCGCTTACAGCTTC     300
CH31    GTTATCGAAGCTTATGACTGGGAATTCATTAAGTCACTGTTTAAGCGCGCTTACAGCTTC     300

CA      AAGTTCCGCTTTAAGTCATTTTTGGGTGCCTACAAGTACTACACTTCCTACACCCTAAAG     360
CH31    AAGTTCCGCTTTAAGTCATTTTTGGGTGCCTACAAGTACTACACTTCCTACACCCTAAAG     360

CA      ACCTTCGACGGTCGTCGTTACTTGGAGCGCTTCGAAGATCGCGTGTCGATGACTGCGCTT     420
CH31    ACCTTCGACGGTCGTCGTTACTTGGAGCGCTTCGAAGATCGCGTGTCGATGACTGCGCTT     420

CA      TTCCTCGCGGACGGTGACACCGCAGTTGCCGAAAGCATGGTTGATGAAATCATGTCGGGT     480
CH31    TTCCTCGCGGACGGTGACACCGCAGTTGCCGAAAGCATGGTTGATGAAATCATGTCGGGT     480

CA      CGCTTCCAGCCAGCAACCCCAACCTTCCTCAATGCTGGCAAGGCACAGCGTGGCGAGCTT     540
CH31    CGCTTCCAGCCAGCAACCCCAACCTTCCTCAATGCTGGCAAGGCACAGCGTGGCGAGCTT     540

CA      GTTTCCTGCTTCCTGTTGCGCATCGAAGACAACATGGAATCCATCGGACGCGCTATTAAC     600
CH31    GTTTCCTGCTTCCTGTTGCGCATCGAAGACAACATGGAATCCATCGGACGCGCTATTAAC     600

CA      TCTTCCCTGCAGCTGTCCAAGCGCGGCGGTGGCGTTGCACTGTTGCTGAGCAACATCCGC     660
CH31    TCTTCCCTGCAGCTGTCCAAGCGCGGCGGTGGCGTTGCACTGTTGCTGAGCAACATCCGC     660

CA      GAATCCGGTGCGCCAATTAAGCACATTGAAAACCAGTCTTCGGGCATCATCCCGATCATG     720
CH31    GAATCCGGTGCGCCAATTAAGCACATTGAAAACCAGTCTTCGGGCATCATCCCGATCATG     720

CA      AAGATGCTGGAAGACGCATTTTCTTACGCTAACCAGCTGGGTGCGCGTCAGGGCGCCGGT     780
CH31    AAGATGCTGGAAGACGCATTTTCTTACGCTAACCAGCTGGGTGCGCGTCAGGGCGCCGGT     780

CA      GCAGTGTACTTGAACGCTCACCACCCAGATATCTTGAACTTCCTCGACACCAAGCGCGAA     840
CH31    GCAGTGTACTTGAACGCTCACCACCCAGATATCTTGAACTTCCTCGACACCAAGCGCGAA     840

CA      AACGCTGACGAGAAGATCCGCATCAAGACCTTGTCTTTGGGAATTGTCATTCCAGACATC     900
CH31    AACGCTGACGAGAAGATCCGCATCAAGACCTTGTCTTTGGGAATTGTCATTCCAGACATC     900

CA      ACCTTCGACCTGGCTAAGCGCAATGAAGATATGTACCTGTTTAGCCCTTATGACGTTGAG     960
CH31    ACCTTCGACCTGGCTAAGCGCAATGAAGATATGTACCTGTTTAGCCCTTATGACGTTGAG     960

CA      CGCGTCTACGGCAAGCCATTTGGTGATATCTCCGTTTCTGATCACTACGCAGAAATGGTC    1020
CH31    CGCGTCTACGGCAAGCCATTTGGTGATATCTCCGTTTCTGATCACTACGCAGAAATGGTC    1020

CA      GAAGACCCACGCATTACTAAGAAGAAGATCAACGCACGTCACTTCTTCCAGACCGTTGCT    1080
CH31    GAAGACCCACGCATTACTAAGAAGAAGATCAACGCACGTCACTTCTTCCAGACCGTTGCT    1080
```

```
CA      GAGCTGCAGTTCGAATCCGGTTACCCATACATCATGTTTGAGGACACCGTAAACAATGCG   1140
CH31    GAGCTGCAGTTCGAATCCGGTTACCCATACATCATGTTTGAGGACACCGTAAACAATGCG   1140

CA      AACCCAGTAAAGACTGGTCGCATCAACATGTCGAACCTGTGCTCCGAGATTCTGCAGGTC   1200
CH31    AACCCAGTAAAGACTGGTCGCATCAACATGTCGAACCTGTGCTCCGAGATTCTGCAGGTC   1200

CA      AACTCCCCATCCTCTTTCAACGATGACTTGAGCTACGAGGAAATGGGCAGTGACATCTCC   1260
CH31    AACTCCCCATCCTCTTTCAACGATGACTTGAGCTACGAGGAAATGGGCAGTGACATCTCC   1260

CA      TGCAACCTCGGCTCCATGAACATTGCTTTGGCGATGGATTCTCCAGACTTCGGTGGCACG   1320
CH31    TGCAACCTCGGCTCCATGAACATTGCTTTGGCGATGGATTTTCCAGACTTCGGTGGCACG   1320

CA      GTCGAAGCTGCTATTCGTGGCCTGACCGCAGTAGCGGACAAGACCGCTATCGATTCCGTC   1380
CH31    GTCGAAGCTGCTATTCGTGGCCTGACCGCAGTAGCGGACAAGACCGCTATCGATTCCGTC   1380

CA      CCATCGATCCGCCACGGCAATGACCAGTCTCATGCCATCGGCCTGGGCCAGATGAACCTC   1440
CH31    CCATCGATCCGCCACGGCAATGACCAGTCTCATGCCATCGGCCTGGGCCAGATGAACCTC   1440

CA      CACGGCTACTTGGGCCGCGAGCACATCTACTACGGCTCTGAAGAAGGCTTGGACTTTACC   1500
CH31    CACGGCTACTTGGGCCGCGAGCACATCTACTACGGCTCTGAAGAAGGCTTGGACTTTACC   1500

CA      AATGCTTACTTCGCGGCAGTTTTGTACGCAGCACTGCGTGCTTCCAACAAGATTGCCAAG   1560
CH31    AATGCTTACTTCGCGGCAGTTTTGTACGCAGCACTGCGTGCTTCCAACAAGATTGCCAAG   1560

CA      GAGCGCGGCGAGACCTTTAGTGAGTTTAAGGACTCCGACTACGCTTCTGGCGTATTCTTC   1620
CH31    GAGCGCGGCGAGACCTTTAGTGAGTTTAAGGACTCCGACTACGCTTCTGGCGTATTCTTC   1620

CA      GATAACTACGATCCAGCAGAGTTTGCACCACAGACCGATAAGGTCAAGGAGCTTTTCGCT   1680
CH31    GATAACTACGATCCAGCAGAGTTTGCACCACAGACCGATAAGGTCAAGGAGCTTTTCGCT   1680

CA      AACTCCACAATCCACACTCCAAGTGCGGAAGACTGGGCAGATCTAAAGGCTGAGGTTATG   1740
CH31    AACTCCACAATCCACACTCCAAGTGCGGAAGACTGGGCAGATCTAAAGGCTGAGGTTATG   1740

CA      GAGCACGGTCTCTACAACCGCAACCTGCAGGCAGTTCCACCAACCGGTTCGATCTCCTAC   1800
CH31    GAGCACGGTCTCTACAACCGCAACCTGCAGGCAGTTCCACCAACCGGTTCGATCTCCTAC   1800

CA      ATCAACAACTCTACTTCCTCGATTCACCCAATCGCTTCCAAGATTGAGATTCGCAAGGAA   1860
CH31    ATCAACAACTCTACTTCCTCGATTCACCCAATCGCTTCCAAGATTGAGATTCGCAAGGAA   1860

CA      GGCAAGATTGGTCGTGTCTACTACCCAGCTCCACACATGGACAACGACAACCTTGATTAC   1920
CH31    GGCAAGATTGGTCGTGTCTACTACCCAGCTCCACACATGGACAACGACAACCTTGATTAC   1920

CA      TTCCAGGATGCATACGAAGTCGGTCACGAAAAGATCATCGACACCTATGCAGTTGCTACG   1980
CH31    TTCCAGGATGCATACGAAGTCGGTCACGAAAAGATCATCGACACCTATGCAGTTGCTACG   1980

CA      AAGTACGTCGACCAGGGCTTGTCTTTGACCTTGTTCTTCAAGGACACCGCGACCACCCGC   2040
CH31    AAGTACGTCGACCAGGGCTTGTCTTTGACCTTGTTCTTCAAGGACACCGCGACCACCCGC   2040

CA      GACATCAACCGCGCGCAGATCTACGCATGGCGCAAGGGCATTAAGACCTTGTACTACATC   2100
CH31    GACATCAACCGCGCGCAGATCTACGCATGGCGCAAGGGCATTAAGACCTTGTACTACATC   2100

CA      CGCTTGCGTCAGATGGCGCTGGCTGGAACCGAGATTGAAGGTTGCGTCTCCTGCATGCTC   2160
CH31    CGCTTGCGTCAGATGGCGCTGGCTGGAACCGAGATTGAAGGTTGCGTCTCCTGCATGCTC   2160

CA      TAA   2163
CH31    TAA   2163
```

Fig. 3.16 Nucleotide sequencing alignment of *nrdE* genes from the parent strain *C. ammoniagenes* ATCC 6872 (CA) and the ts-mutant CH31. Nucleotide substitution is highlighted.

Both nucleotide sequences were translated to their corresponding amino acids according to the codon usage in corynebacteria (Malumbres *et al.* 1993). From the alignment of the deduced amino acid sequences (Fig. 3.17), it can be noticed that the point mutation in the nucleotide position 1301 led to a codon alteration from UCU to UUU resulting in substitution at amino acid position 434 from serine (S) to phenylalanine (F). This amino acid substitution was abbreviated as S434F.

```
CA      VTQQLGKTVAEPVKNSEKLDFHALNALLNLYDEDGKIQFDKDREAANQYFLQHVNQNTVY   60
CH31    VTQQLGKTVAEPVKNSEKLDFHALNALLNLYDEDGKIQFDKDREAANQYFLQHVNQNTVY   60

CA      FHDLEEKIEYLVENKYYEPEVIEAYDWEFIKSLFKRAYSFKFRFKSFLGAYKYYTSYTLK   120
CH31    FHDLEEKIEYLVENKYYEPEVIEAYDWEFIKSLFKRAYSFKFRFKSFLGAYKYYTSYTLK   120

CA      TFDGRRYLERFEDRVSMTALFLADGDTAVAESMVDEIMSGRFQPATPTFLNAGKAQRGEL   180
CH31    TFDGRRYLERFEDRVSMTALFLADGDTAVAESMVDEIMSGRFQPATPTFLNAGKAQRGEL   180

CA      VSCFLLRIEDNMESIGRAINSSLQLSKRGGGVALLLSNIRESGAPIKHIENQSSGIIPIM   240
CH31    VSCFLLRIEDNMESIGRAINSSLQLSKRGGGVALLLSNIRESGAPIKHIENQSSGIIPIM   240

CA      KMLEDAFSYANQLGARQGAGAVYLNAHHPDILNFLDTKRENADEKIRIKTLSLGIVIPDI   300
CH31    KMLEDAFSYANQLGARQGAGAVYLNAHHPDILNFLDTKRENADEKIRIKTLSLGIVIPDI   300

CA      TFDLAKRNEDMYLFSPYDVERVYGKPFGDISVSDHYAEMVEDPRITKKKINARHFFQTVA   360
CH31    TFDLAKRNEDMYLFSPYDVERVYGKPFGDISVSDHYAEMVEDPRITKKKINARHFFQTVA   360

CA      ELQFESGYPYIMFEDTVNNANPVKTGRINMSNLCSEILQVNSPSSFNDDLSYEEMGSDIS   420
CH31    ELQFESGYPYIMFEDTVNNANPVKTGRINMSNLCSEILQVNSPSSFNDDLSYEEMGSDIS   420

CA      CNLGSMNIALAMD[S]PDFGGTVEAAIRGLTAVADKTAIDSVPSIRHGNDQSHAIGLGQMNL   480
CH31    CNLGSMNIALAMD[F]PDFGGTVEAAIRGLTAVADKTAIDSVPSIRHGNDQSHAIGLGQMNL   480

CA      HGYLGREHIYYGSEEGLDFTNAYFAAVLYAALRASNKIAKERGETFSEFKDSDYASGVFF   540
CH31    HGYLGREHIYYGSEEGLDFTNAYFAAVLYAALRASNKIAKERGETFSEFKDSDYASGVFF   540

CA      DNYDPAEFAPQTDKVKELFANSTIHTPSAEDWADLKAEVMEHGLYNRNLQAVPPTGSISY   600
CH31    DNYDPAEFAPQTDKVKELFANSTIHTPSAEDWADLKAEVMEHGLYNRNLQAVPPTGSISY   600

CA      INNSTSSIHPIASKIEIRKEGKIGRVYYPAPHMDNDNLDYFQDAYEVGHEKIIDTYAVAT   660
CH31    INNSTSSIHPIASKIEIRKEGKIGRVYYPAPHMDNDNLDYFQDAYEVGHEKIIDTYAVAT   660

CA      KYVDQGLSLTLFFKDTATTRDINRAQIYAWRKGIKTLYYIRLRQMALAGTEIEGCVSCML   720
CH31    KYVDQGLSLTLFFKDTATTRDINRAQIYAWRKGIKTLYYIRLRQMALAGTEIEGCVSCML   720
```

Fig. 3.17 Amino acid identity between the NrdE proteins (R1E) from both the parent strain *C. ammoniagenes* ATCC 6872 (CA) and the ts-mutant CH31. The exchanged amino acid is highlighted.
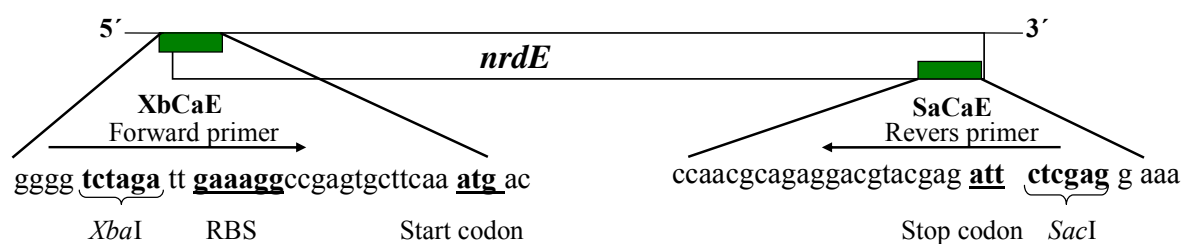
3.2.4 Genetic complementation of the ts-mutant CH31 with *nrdE*⁺ of *C. ammoniagenes* ATCC 6872

The base pair substitution detected in the *nrdE*^{ts} gene at nt 1301 raised the important question whether this point mutation is responsible for the thermosensitivity of the mutant CH31. Therefore, it was necessary to complement the mutant phenotype of the strain CH31 with the wild-type *nrdE*⁺ gene of the parent strain.

3.2.4.1   Construction of pXE6872 and pXECH31 plasmids

In order to avoid any effect of flanking regions only the complete *nrdE*⁺ gene together with its putative ribosome binding site (RBS) should be introduced into the ts-mutant CH31. A successful complementation would be noticeable by growth at the non-permissive temperature due expression of functional NrdE. For high expression of the desired genes (*nrdE*⁺ or *nrdE*^{ts}) in coryneform bacteria *C. glutamicum/E. coli* shuttle vector pXMJ19 (Jakoby *et al.* 1999) has been recommended (Bott 2001). However, the intended cloning of the *nrdE*⁺ or *nrdE*^{ts} genes from pUCEF6872 or pUCEFCH31, respectively, into pXMJ19 was confronted with the absence of suitable restriction sites flanking the desired gene. New restrictions sites were added to the *nrdE*⁺ or *nrdE*^{ts} genes by PCR using primers XbCaE and SaCaE. The forward primer XbCaE matched the RBS located 14 nucleotides upstream of *nrdE* gene, followed by the complete sequence of *nrdE* (Fig. 3.18).This primer also changed the start codon from GTG to ATG (the known start codon in *C. glutamicum*). The reversed primer SaCaE corresponded to the end of *nrdE* including the TAA stop codon. These two designed primers were used for PCR-amplification of *nrdE*⁺ gene from the wild-type strain *Corynebacterium ammoniagenes* ATCC 6872. Likewise, the mutated *nrdE* gene of the ts-mutant CH31 was amplified using the same designed-primers and was used in the complementation experiment as a negative control.

Fig. 3.18 Sequence of the expression primers designed for PCR-amplification and cloning of the *nrdE*$^+$ and *nrdE*^{ts} genes (from wild-type ATCC 6872 and ts-mutant CH31, respectively) which used in genetic complementation test. XbaCaE is the front primer at the 5´-end containing *Xba*I restriction site; while SaCaE is the reverse primer at the 3´-end contained *Sac*I restriction site

To simplify the construction of the *nrdE* expression vectors, the *taq* polymerase-amplified PCR products were cloned in the plasmid vector pCR®2.1TOPO. The recombinant plasmids were amplified *in vivo* in *E. coli* Top10F´. One plasmid containing the *nrdE*^{ts} gene (pTopCH31) was checked carefully by sequencing. The amplified *nrdE*^{ts} gene was found to be identical with that of the ts-mutant CH31. Subsequently, the plasmid pTopCH31 was digested with *Xba*I and *Sac*I, the 2.2 kb fragment including the *nrdE*^{ts} was separated, purified and ligated into the shuttle vector pXMJ19, digested with the same enzymes. The constracted plasmid was transformed into *E. coli* and named pXECH31.

In order to construct an additional expression vector containing the *nrdE*$^+$ gene the plasmid pXECH31 was treated with *Eco*4-III and *Nsi*I. The 1.544 kb *Eco*4-III-*Nsi*I-fragment from pXECH31 containing the point mutation was replaced by the same fragment from the wild-type gene, isolated from plasmid pUCEF6872 (Fig. 3.19).
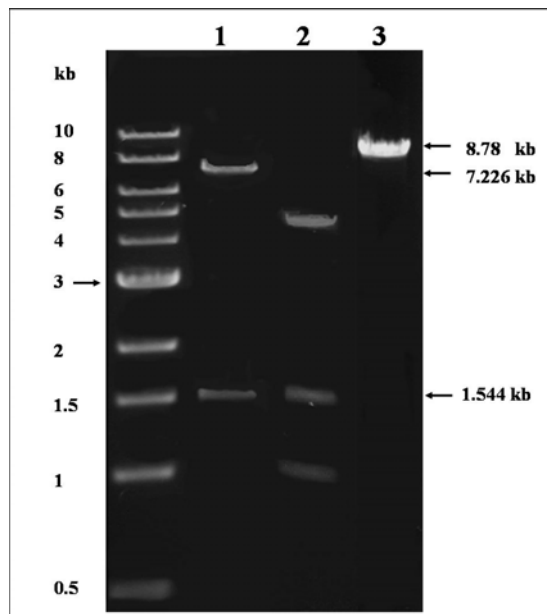
Fig. 3.19 Electrophoresis analysis of pXECH31 (lane 1) and pUCEF6872 (lane 2) digested with *Eco*47-III and *Nsi*I. The recombinant vector pXE6872 resulted after successful ligation of 1.544 kb fragment from pUCEF6872 with 7.226 kb fragment from pXECH31 was presented in lane 3.

3.2.4.2   *Overexpression of nrdE$^+$ and nrdE^{ts} genes in the ts-mutant CH31*

The original vector pXMJ19 or its derivative pXE6872 and pXECH31 were introduced into *E. coli* XL1-Blue. Since expression of *nrdE* cloned in the shuttle vector pXMJ19 is under control of the *tac* promotor, 1 mM IPTG was used to induce *nrdE* expression (Sec. 2.6.2.2). The total extracted proteins were run in a SDS-PAGE gel and either stained with Coomassie Blue or immu-nostained with rabbit polyclonal antiserum (Rb-anti-CEDAF, #2778, Sec. 2.1) raised against *nrdE* from *C. ammoniagenes*. In these experiments, a cross-reacting polypeptide of about 81 kDa was detected in the IPTG-induced *E. coli* cells carrying pXE6872 and pXECH31, but was absent in non-induced cells carrying the same plasmids. Moreover, the cross-reacting polypeptide was not detected in *E. coli*/pXMJ19.

After successful expression of both *nrdE$^+$* and *nrdE^{ts}* in *E. coli*, pXE6872 and pXECH31 were isolated and introduced into the ts-mutant CH31 cells via

electroporation (Sec. 2.5.13.2). The ts-mutant CH31 cells complemented with either $nrdE^+$ (CH31/pXE6872) or $nrdE^{ts}$ (CH31/pXECH31) did not grow at 37°C without pre-induction with IPTG. To determine pre-induction period required for displaying functional expression of $nrdE$ in ts-mutant CH31, the complemented cells were treated with 1 mM IPTG for 2, 4, or 6 h at 27°C in NBH-broth containing 30 µg / ml Cm and 1mM IPTG (Sec. 2.5.16). At all induction periods the CH31/pXECH31 cells did not grow at 37°C, whereas the same cells grew at 27°C up to 10^7 cfu / ml. On the other hand, CH31/pXE6872 did not grow at 37°C without induction, but grew at 37°C up to level of 10^6 cfu / ml after 2 h induction with 1 mM IPTG. The cell viability of CH31/pXE6872 was reached to 10^7 cfu / ml at 37°C when the cells were pre-induced for 4 or 6 h before plating (Fig. 3.20). Moreover, the colonies of the strain CH31/pXE6872 which were induced for 4 or 6 h showed better growth compared with that were induced for only 2 h. According to these results the complemented CH31 cells were induced with 1 mM IPTG for 4h before studying the biological activity of the expression of $nrdE^+$ or $nrdE^{ts}$ in the ts-mutant CH31.
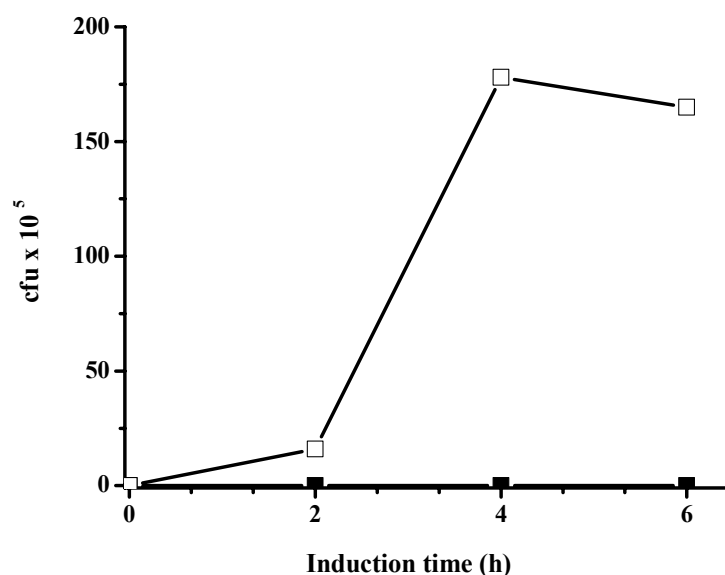
Fig. 3.20 Delayed recovery of cell viability at 37°C of the ts-mutant CH31 complemented with $nrdE^+$ (cloned in pXE6872) or $nrdE^{ts}$ (cloned in pXECH31) after pre-induction at 27°C with 1mMI PTG.

The overexpression of the cloned genes ($nrdE^+$ or $nrdE^{ts}$) in the ts-mutant CH31 was demonstrated both by Coomassie staining and immunoblotting using the specific antibody (Fig. 3.21). An enormously high level of expression of both NrdE$^+$ and NrdE^{ts} was observed after 4 h induction with 1 mM IPTG. Clearly, a cross-reacting polypeptide of a bout 81 kDa was detected in the IPTG-induced CH31/pXE6872 and CH31/pXECH31, but was absent in non-induced cells carrying the same plasmids.
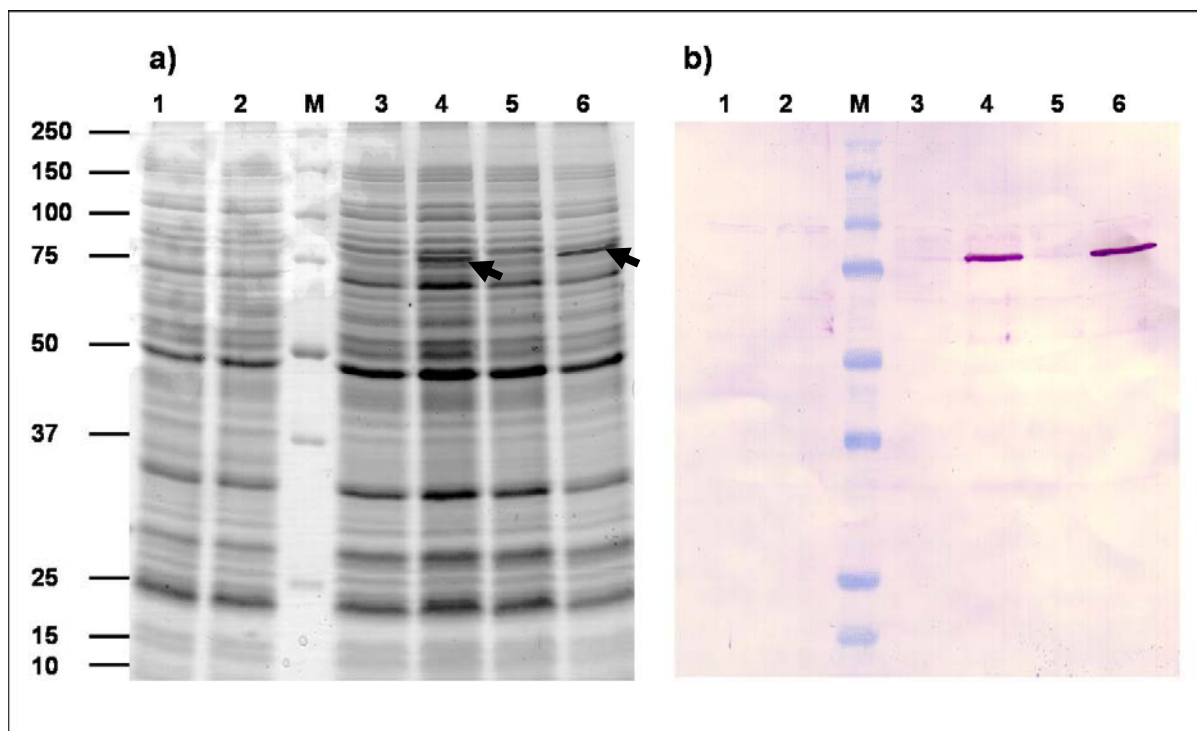
Fig. 3.21 Expression of $nrdE^+$ (from wild-type strain ATCC 6872) and $nrdE^{ts}$ (from the ts-mutant CH31) in.the ts-mutant CH31. Strain CH31 carrying pXMJ19 (lanes 1 and 2), CH31 carrying pXE6872 (lanes 3 and 4), and CH31 carrying pXECH31 (lanes 5 and 6) were grown in NBH-broth in the presence of 1 mM IPTG for 4 h (lanes 2, 4, and 6) or in the absence of IPTG (lanes 1, 3, and 5). The panel on the left (a) shows SDS-PAGE gel of the total protein extracts, the panel on the right (b) shows the western blot probed with ant-$nrdE$ antibody (2778). Protein standards (Bio-Rad #161-0373) were run in lane M.

To determine whether the overexpressed products (NrdE$^+$ and NrdE^{ts}) of the cloned genes displayed biological activity in the ts-mutant CH31, the growth of complemented cells was examined at the permissive (27°C) and the non-permissive (37°C) temperatures. As described above the examined cultures were pre-induced with 1 mM IPTG at 27°C for 4 h (Sec. 2.5.16). After 3 days, the strain CH31/pXMJ19 grew at 27°C, while the shuttle vector pXMJ19 carried the *cm^r* gene, but did not grow at 37°C. Likewise, the strain CH31/pXECH31 displayed normal growth at 27°C, but its growth was totally absent at 37°C (Fig. 3.22), although the high level expression of the defect NrdE^{ts} (Fig. 3.21). Obviously, only the ts-mutant CH31 complemented with the healthy *nrdE$^+$* gene from the wild-type ACCT 6872 grew at 27 and 37°C (Fig. 3.22).
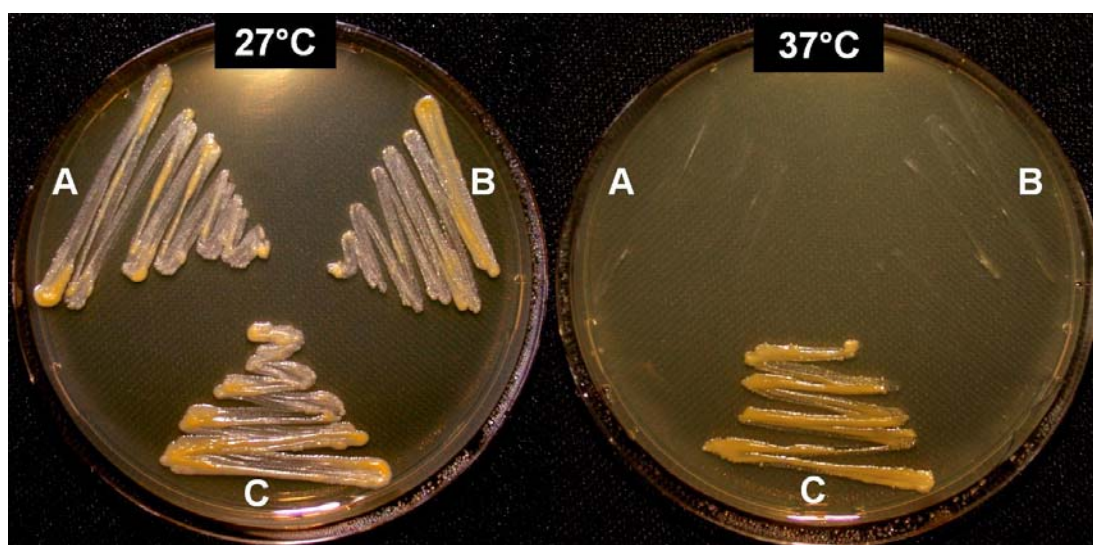
Fig. 3.22 Growth of the ts-mutant CH31 carrying pXMJ19 (A), pXECH31 (B), and pXE6872 (C) after 3 days onto NBH agar supplemented with 1mM IPTG in the presence of 30 µg / ml chloramphenicol.

Morphologically, the complemented cells of the strain CH31/pXE6872 grew normally at the permissive (27°C) and the non-permissive (37°C) temperatures. The grown cells did not display any morphological changes at both temperatures (Fig. 3.23).

2 h at 27°C   2 h at 37°C
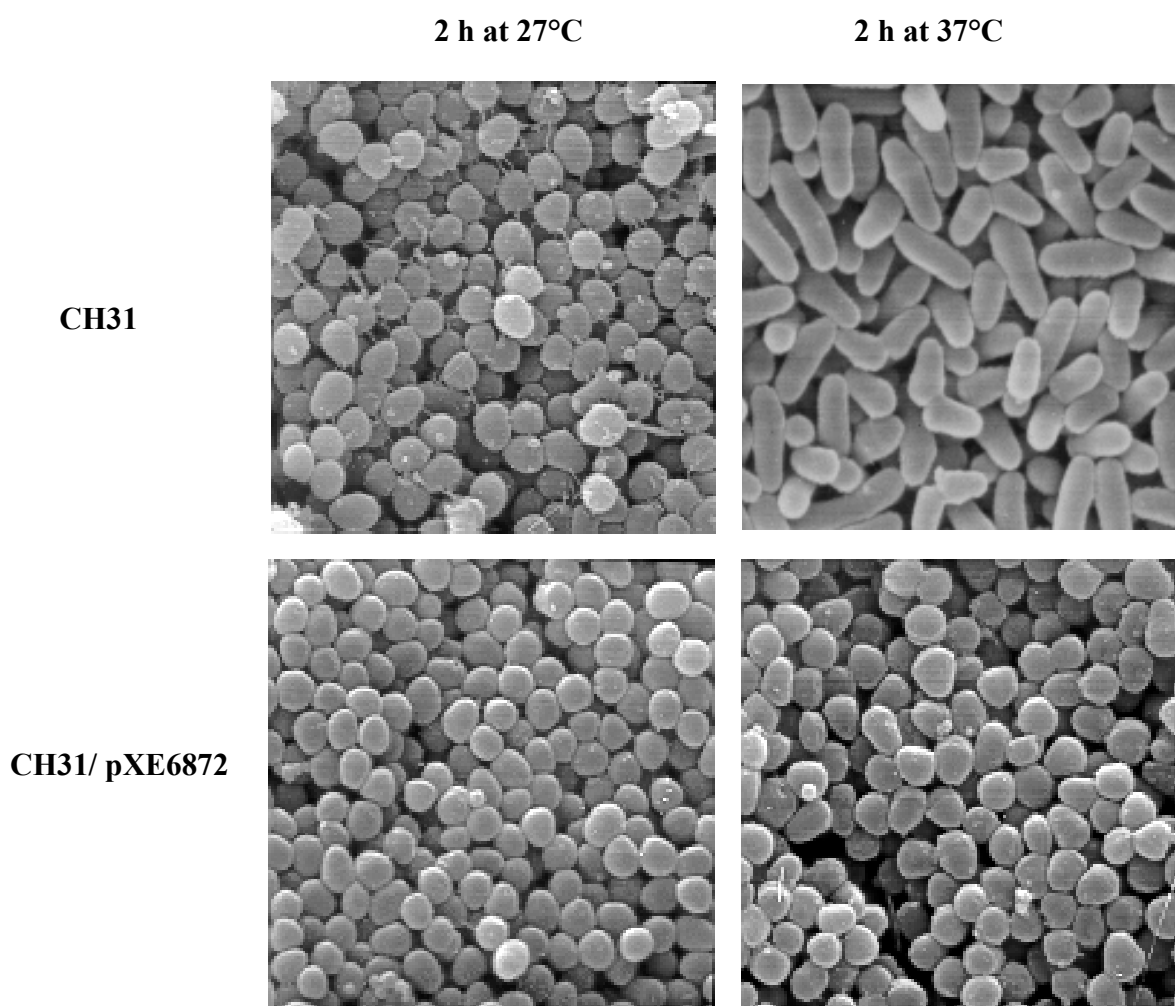
CH31

CH31/ pXE6872

Fig. 3.23 Morphology of the ts-mutant CH31 complemented with the *nrdE*$^+$ carried in pXE6872 grew at the permissive (27°C) and non-permissive (37°C) temperatures. The elongated cells of the ts-mutant CH31 which was grown at 37°C were presented for comparison.

3.3 Accumulation of IMP by the ts-mutant CH31

3.3.1 Ability of strain CH31 to accumulate IMP extracellularly

After the successful application of the ts-mutant CH31 in the production of NAD$^+$ the extracellular accumulation of inosine 5′-mono-phosphate (IMP), as a commercial flavor enhancer, was also investigated. The ability of the ts-mutant CH31 to produce IMP was examined in the synthetic fermentation me-

dium supplemented with 10 μM MnCl$_2$ as described in Materials & Methods (Sec. 2.6.5.3). The unbalanced growth of the ts-mutant CH31 induced by temperature shift to 37°C was observed by the continued increasing of cell biomass (from 5.4 to 11.3 g / l, before and 24 h after shifting the temperature to 37°C, respectively) accompanied by dramatic reduction in the cell counts (Fig. 3.24). Under these conditions of unbalanced growth, the shifted culture of the ts-mutant CH31 had ability to accumulate IMP. Remarkably, threefold increase in accumulation of IMP (0.46 g / l) was detected by shifted culture compared with the control culture which was held under conditions of the balanced growth (0.16 g / l). The maximum accumulated amount of IMP was obtained on the fifth day of fermentation at 37°C. However, this accumulated amount of IMP was unstable on the following days. Therefore, the IMP protocol was modified by the daily adjustment of the pH of cultivation broth at 7.5 - 8.0 on the third day of cultivation. This modification in the cultivation conditions led to enhance the stability of the accumulated IMP during two days after detection of the maximum accumulation. Therefore the further experiments for the production of IMP were done with adjustment of pH at 7.5 – 8.0 as described in Materials & Methods (Sec. 2.6.5.3)
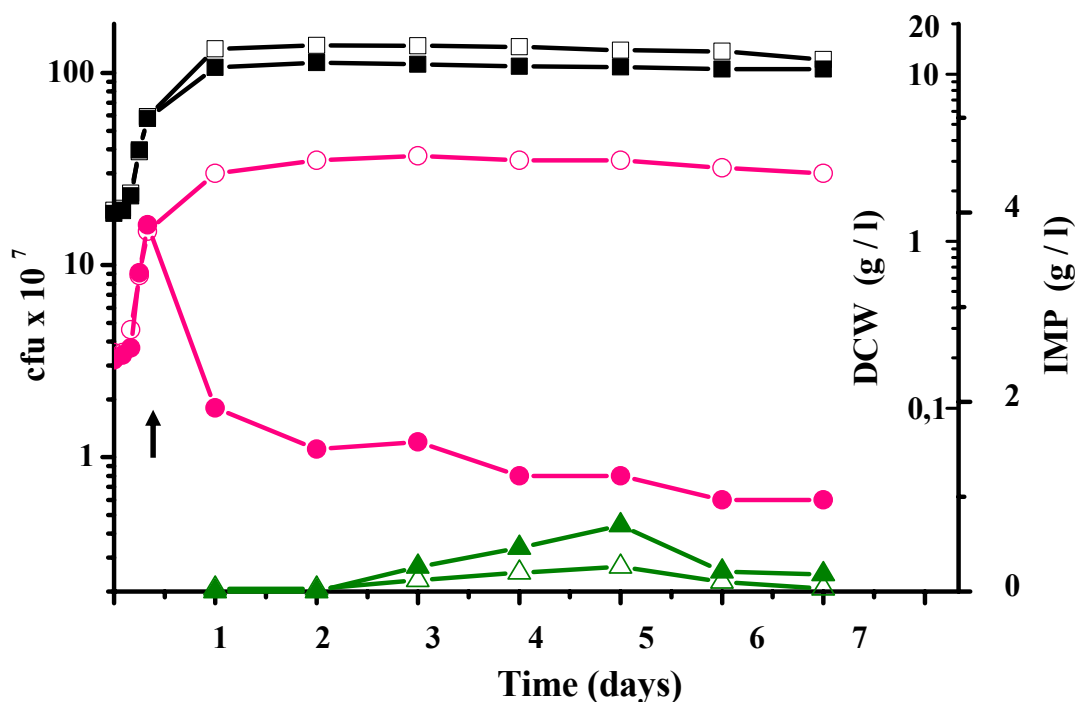
Fig. 3.24 IMP overproduction (▲) of unbalanced grown *nrd* ^{ts} mutant CH31 upon transfer to 37°C (full symbols) versus balanced growth when kept at 27°C (empty symbols). The Mn^{2+}-supplied fermentation medium of Nakayama *et al.* (1968), modified according to Teshiba and Furuya (1982) was used. Viability was examined by colony counting (cfu, ●) and cell growth was recorded by OD_{578} and re-calculated as dry cell weight (■) from a standard curve previously prepared. The arrow indicates time of temperature shift.

The morphological changes of the ts-mutant CH31 cells under condition of the unbalanced growth in fermentation medium due to incubation at the non permissive temperature (37°C) was investigated by SEM (Fig. 3.25). Since the fermentation medium is basically a mineral medium growth slower than in complex medium was expected. Therefore, the cell morphology was investigated in fermentation medium 24 h after the temperature shift. Beside induction of limited elongation, the inhibited cells grown in fermentation medium became fat.
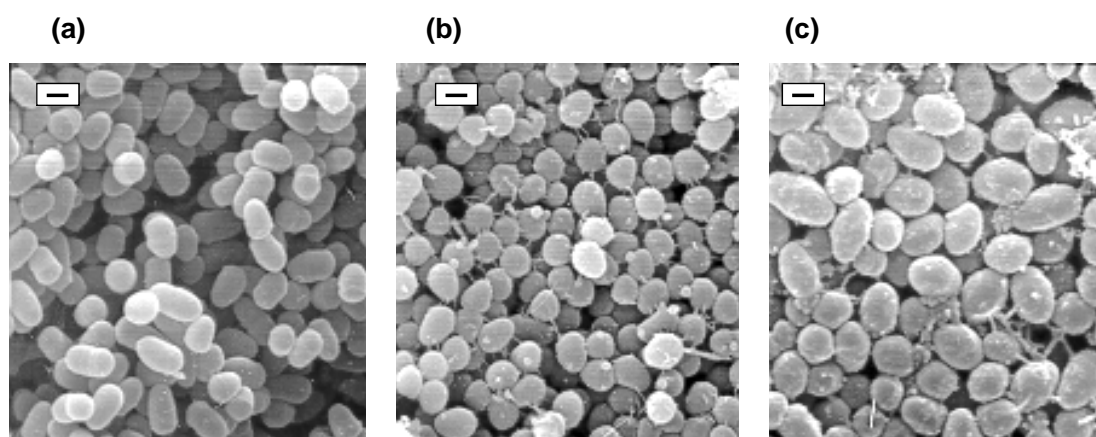
Fig. 3.25 Morphological changes ''Cells get fat'' of the ts-mutant CH31 cells grown in fermentation medium upon temperature shift to 37°C (c) versus cells kept at 27°C (b), which has entered the stationary phase of growth after 24 hours. Cells from the logarithmic phase of growth (7.5 h-old) just before the temperature shift are shown for comparison (a). The bar represents 1 μm.

3.3.2 Enhancement of IMP accumulation of strain CH31 by salvage pathway

Nucleotides may be formed in microorganisms by *de novo* biosynthesis or salvage of preformed bases and interconversion to the desired compound (Kulkarni *et al.* 1998). After successful accumulation of IMP by the ts-mutant CH31 through *de novo* biosynthesis pathway, hypoxanthine (Hx) was used as precursor for IMP accumulation through salvage mechanism. Experimentally, hypoxanthine (2.5 g / l) was added to the cultivation broth just after temperature shift to 37°C. Salvage accumulation of IMP started one day earlier (on the second day, Fig. 3.26) compared to accumulation by *de novo* synthesis only (Fig. 3.24). Likewise, under salvage conditions the maximum of IMP accumulation (3.85 g / l) was detected one day earlier (on the fourth day). Moreover, the adjustment of pH seemed to be correlated with reduced degradation of accumulated IMP, i. e., the amount of IMP detected at the fifth and sixth day of cultivation was near to the maximum of IMP production. Generally, the IMP productivity of the ts-mutant CH31 was remarkably enhanced (about tenfold)

compared with that of the control culture at 27°C (Fig. 3.26). The behavior of cells during cultivation toward consumption of hypoxanthine was also determined. In the control culture, the amount of hypoxanthine decreased slightly during cultivation from 2.5 g to 1.98 g per liter within four days. On the other hand, the amount of hypoxanthine in the productive culture at 37 °C was as low as 1.6 g per liter after only two days and decreased sharply to 0.6 g per liter simultaneously with the increase of IMP accumulation to a maximum (Fig. 3.26).
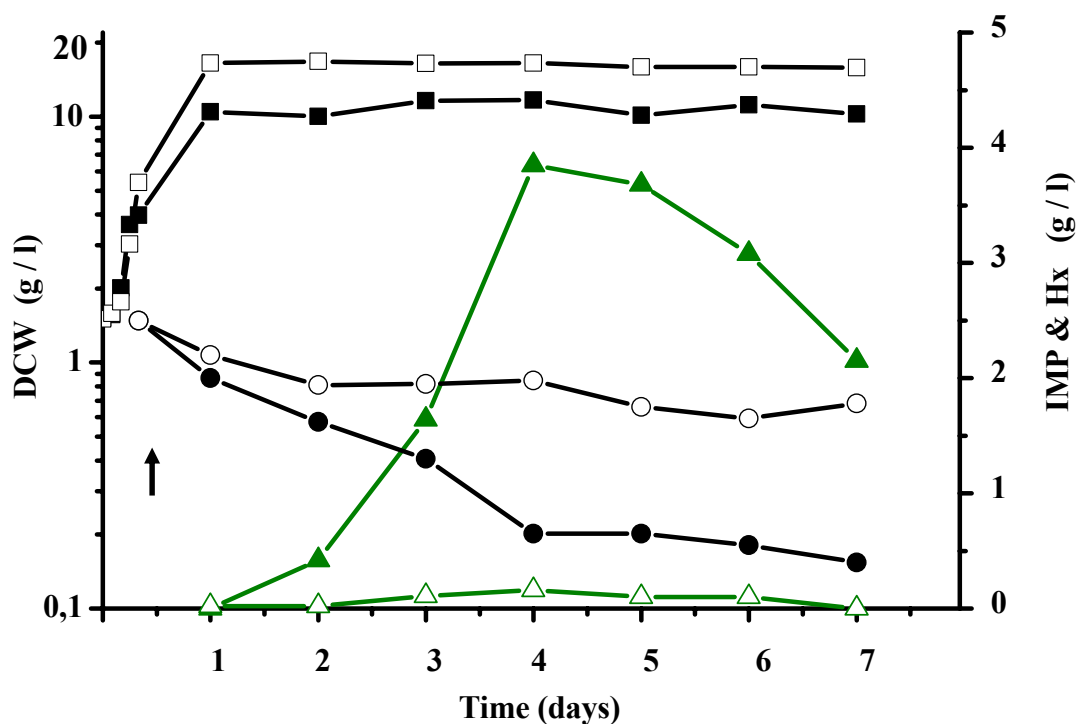

Fig. 3.26 Salvage accumulation of IMP (▲) by *nrd* ^{ts} mutant CH31 induced by shifting the temperature to the non-permissive temperature (37°C, arrow) and addition of 2.5 g hypoxanthine per liter (Hx, ●). Open symbols refer to the control experiment which achieved without temperature shift. Cell growth was expressed as dry weight (■) as described in Fig. 3.24.

3.3.3 Optimization of IMP production

The conditions of IMP production were optimized firstly in baffled flasks and then the optimum conditions were applied at large scale in 10-liter bioreactor. The effect of glucose feeding on the accumulation of IMP by the ts-mutant CH31 was examined. Forty percent of glucose (52 g) was added and sterilized with the other components of the fermentation medium. The remained glucose amount (60%) was sterilized separately and added intermediately on the second and third day of cultivation, 30% each time (39 g / l). Figure 3.27 shows that the applied feeding system had a simulative effect not only for the early accumulation of IMP but also for product stability. The maximum level of IMP (3.6 g) was detected on the third day. Moreover, similar amounts were obtained during the next three days. On the other hand, about 80% of the hypoxanthine amount was quickly used by the ts-mutant CH31 cells in the first stage of cultivation (three days) at 37°C compared with the control culture in which the glucose was altogether added to the medium and sterilized with the other components (Fig. 3.27a). Another experiment was carried out without addition of the last batch of glucose (39 g, on the third day). Similar data were obtained when the third amount of the glucose (30%), was omitted. Therefore, the further experiments were done using only 70% (91 g / l) of the total amount of glucose, 52 g / l during preparation of the cultivation medium and 39 g / l on the second day (separately autoclaved).

As a natural nutrient, meat or yeast extract has been used in the production of several nucleic acid products (Kotani *et al.* 1978). However it is desirable to find a cheaper and more simulative natural nutrient for IMP accumulation. In this study, corn steep liquor (10, 15, or 20 g) was substituted for yeast extract in the fermentation medium. Because of the inhibitory effect of the high concentrations of the corn steep liquor (CSL) on the growth of cells, 15 g CSL per liter were used to incorporation in the fermentation medium for the production of IMP (Fig. 3.27b).

(A)

(B)
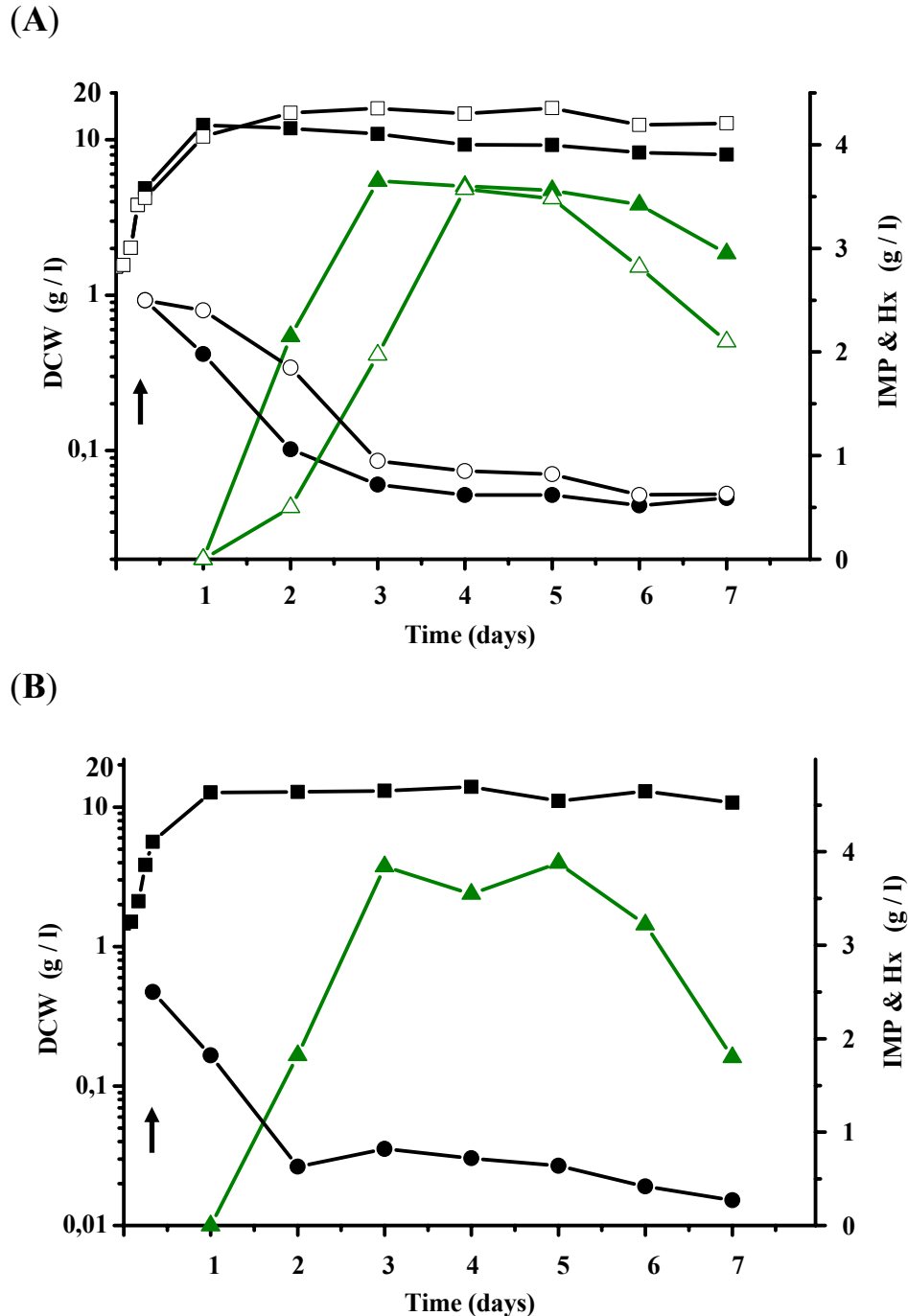
Fig 3.27 (A) Accumulation of IMP (▲) with the ts-mutant CH31 by schedules the glucose feeding (52, 39, and 39 g / l after 0, 24, and 48 h, respectively). Hypoxanthine (Hx, ●) was added at level 2.5 g / l simultaneously with the temperature shift (37°C, arrow). Growth was expressed as dry cell weight (■) as described in Fig. 3.24. Open symbols refer to the control experiment in which the total amount of glucose (130 g / l) was added and sterilized with the other components of the fermentation medium. (B) Accumulation of IMP by the ts-mutant CH31 with replacement the yeast extract with 15 g corn steep liquor, and omitting the third batch of glucose.

The growth and IMP accumulation behaviors of the ts-mutant CH31 were not affected by replacement of the 10 g yeast extract by 15 g corn steep liquor. The maximum accumulated IMP (3.8 g / l) can be detected on the third and fifth days of cultivation. On the other hand, no negative effect on cell growth or hypoxanthine utilization was noticed by replacement of yeast extract with the corn steep liquor. Therefore, in the scaling up experiment for the nucleotide production in 10-liter bioreactor corn steep liquor was used instead of yeast extract in addition to reduction of glucose amount from 130 g to 91 g as described above.

3.3.4   Large scale production of IMP by strain CH31

The main aim of this experiment was to establish controlled and highly reproducible culture conditions for IMP production by the ts-mutant CH31. For this purpose, the optimal IMP-production conditions in flasks (Sec. 3.3.3) were applied to produce IMP in a 10 liter jar bioreactor (Sec. 2.6.5.4). From the preliminary experiments the scaling up of the nucleotide production protocol faced several problems. Due to the relative high lipid content of the cell wall of corynebacteria, they form aggregates in liquid culture. In addition, foam formation was the second problem facing nucleotide production with corynebacteria in the bioreactor, especially *Corynebacterium ammoniagenes*. Therefore the final IMP-experiment described in this work was concerned with finding suitable solutions for these problems in order to increase IMP-productivity. Foam formation was prevented by the addition of the antifoam emulsion Desmophen. The use of this antifoam was not sufficient to prevent foam formation. Moreover use of a large amount of this antifoam agent had a negative effect on the growth of cells resulting in reduction of IMP productivity. Therefore, a mechanical method for foam reduction was developed besides using the antifoam emulsion Desmophen, but at low level (ca. 4 ml were added during the five days of cultivation). To prevent or reduce cell aggregation, 0.1% tween 80 was added to the fermentation medium before inoculation with the mother culture (Sec. 2.6.5.4).

Under controlled growth conditions in the bioreactor (Fig. 3.28), 4.33 g IMP per liter was accumulated 32 h after the onset of cultivation (25 h after induction of the unbalanced growth by temperature shift to 37°C). This IMP amount was stable during the next four days when the detected IMP in this period was not less than 3.5 g IMP per liter. The maximum accumulated amount was slightly higher than that produced in the flasks. At the same time, a dramatically reduced amount of hypoxanthine was detected during the 32 h after the onset of cultivation. This behavior was similar to that obtained during nucleotide fermentation in the flasks.

Although the maximum amount of IMP was obtained after 32 h, all parameters were recorded for 5 days to determine the stability of the IMP produced. The pO_2 value was kept at 30% saturation by a combination of pulsed aeration and agitation. These conditions allowed high reproductively of the corynebacterial cultures, as demonstrated in the preliminary experiments. The cultivation period in the bioreactor can be divided into three periods: I, II and III. I-period, from starting the cultivation until temperature shift (37°C) 7.5 h after the onset of cultivation; II-period, from shifting the temperature until obtaining the maximum accumulated IMP (32 h); III-period, from 32 h to the end of cultivation (120). The pH of the bioreactor culture was increased in the I-period from 7.0 to 7.5 and later increased rapidly to 8.5 in the II-period. Afterwards the pH was adjusted to a level 7.5-8.0 as described in Materials & Methods (Sec. 2.6.5.4). In the II-period, decrease in both pO_2 and percentage of the O_2 exhausted was measured accompanied with increaseing in percentage of the CO_2 exhausted (Fig. 3.29). This disturbed period was ended by obtaining the maximum IMP accumulation, after that in the III-period all recorded parameters were stable.
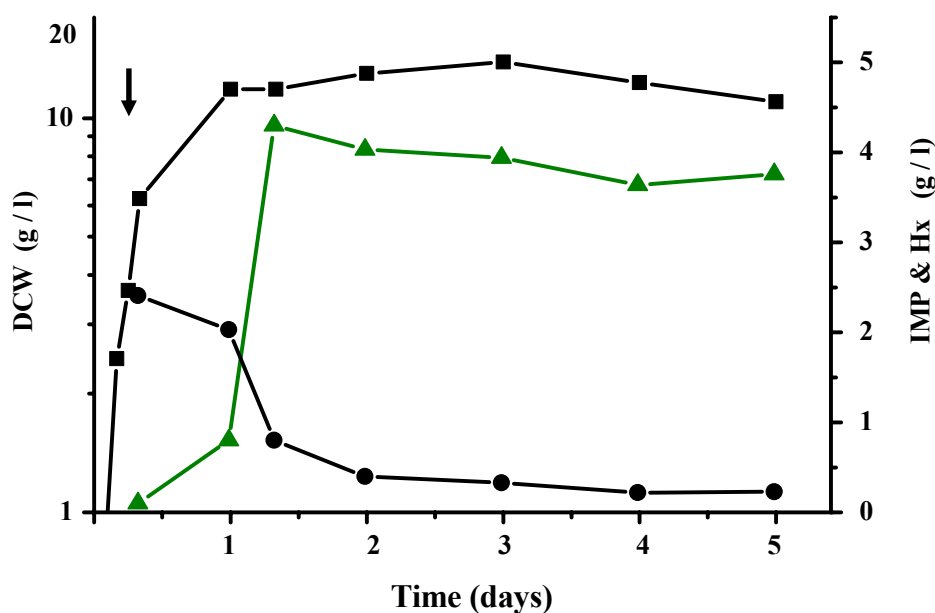
Fig. 3.28 Large scale production of IMP by the ts-mutant CH31 in 10-liter biore-actor (Biostat E). Corn steep liquor was used and the glucose feeding schedule was applied as described in Fig. 3.27A. Symbols: (▲) IMP; (●) Hx; (■) DCW. The ar-row refers to the temperature shift.
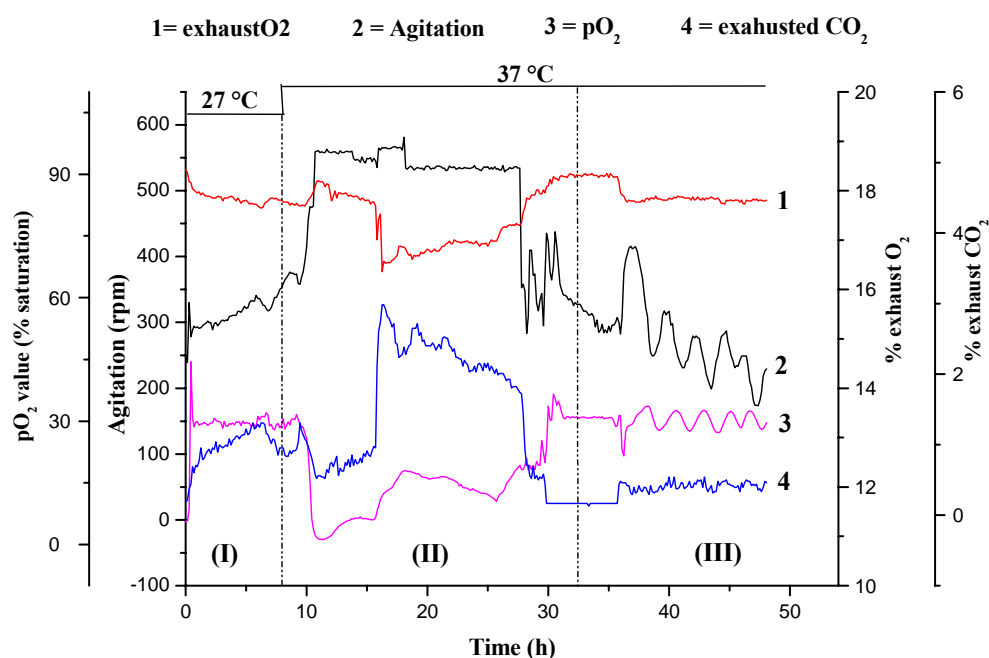
Fig. 3.29 Cultivation parameters of the ts-mutant CH31 culture during cultivation in a 10-liter bioreactor (for more details see the text)
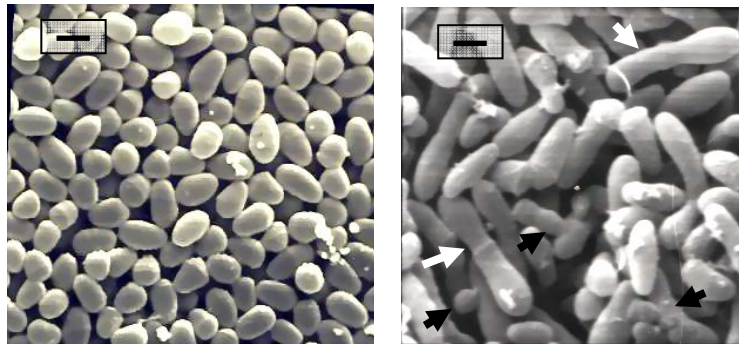
Fig. 3.30 Morphological changes of the 48 h old cells of the ts-mutant CH31 (right) under the non-permissive conditions (37°C) in 10-liter bioreactor. Dumbbell-shaped and T-shaped cells were indicated with white and black arrows, respectively. The normal rods of the ts-mutant CH31 just before temperature shift (7.5 h after the onset of cultivation) were also presented (left).

The morphological changes of the productive cells under controlled growth conditions in the bioreactor were examined by SEM. Sample was taken from the unbalanced culture (at 37°C) after 48 h from the onset of the cultivation. The effect of temperature shift was clearly demonstrated by the induction of limited elongation (2 - 3 times of the normal length of wild-type cells). Dumbbell-shaped rods (3 - 4.5 µl) were observed by the temperature-inhibited culture of the ts-mutant CH31 (Fig. 3.30). Remarkably, the inhibited rods did not indicate any signs of the initiation of septum formation. Moreover, T-shaped cells were observed under the non-permissive conditions

4 DISCUSSION

The modern biotechnology still utilizes traditional techniques beside modern knowledge and techniques to modify, create, or select the particular 'biological process' or 'organism'. This is where the terms 'gene technology', 'genetic engineering' and 'genetic modification' enter the equation (Demain 2000). Thus, studies in this field are concerned not only with increasing the yield of production but also with finding of new strains and development of alternative biological process.

4.1 Correlation between inhibition of cell-cycle of *C. ammoniagenes* and nucleotide production

4.1.1 Direct and indirect inhibition of cell-cycle

Synchronized cultures are the classical tool for cell-cycle studies (B-, C-, and D period, cf. Introduction 1.2). However, it is often overlooked that cell-cycle events may be analyzed simply by studying exponentially growing cells and manipulating them directly or indirectly. For example, addition of certain antibiotics to the latter gives exactly the same result as in the synchrony experiment (Cooper 1991). However, expanding cell-cycle studies from the prokaryotic model *E. coli* (cf. Introduction) with its fast growth rate to further organisms it cannot be ignored that their generation times may differ widely. For instance, arresting the cell-cycle of *Corynebacterium glutamicum* was studied after 2 h of treatment with different antibiotics (Kijima *et al.* 1998). Inhibition of the cell-cycle can be directly effected when certain processes in the cell-division events, such as septum formation, Z-ring, and septation, are inhibited (Trusca, *et al.* 1998). On the other hand, the indirect inhibition of the cell-cycle occurs as a response, for example when DNA replication is blocked (Husiman *et al.* 1984). The microscopic examination of the total population of cells can give information about the division cycle; once again synchronization is not

necessary for to analyzing the cell division cycle.

Inhibition of septum formation is one of the major tools of the bacterial cell-cycle which can be applied as a direct target for inhibition of cell-cycle. The process of cell division or cytokinesis requires spatial, temporal, and quantitative controls so that newly replicated chromosomes are properly partitioned to daughter cells. The spatial control usually involves the identification of the midpoint of the cell where the septum or cleavage furrow will form (cf. Introduction, Fig.1.2). The temporal and quantitative controls ensure that the newly replicated chromosomes are equipartitioned. To induce septation, the Z-ring must bring about invagination of the cytoplasmic membrane, which is accompanied in most bacteria by the synthesis of septal peptidoglycan. These two processes appear to be closely coupled in many bacteria, because inhibitors of septal peptidoglycan biosynthesis block further invagination of the cytoplasmic membrane (Lutkenhaus and Addinall 1997).

Cephalexin is known as a specific inhibitor of *E. coli* cell-cycle by impeding the septum peptidoglycan synthetase, penicillin-binding protein 3 (Spratt 1975), encoded by *ftsI* (Rothfield and Justice 1997). The treatment of *E. coli* with cephalexin led to inhibition of cell-cycle and induction of cell filamentation (Pogliano *et al.* 1997; Gordon *et al.* 1997). This antibiotic was successfully applied for inhibition of cell division and induction of only limited elongation in *Corynebacterium glutamicum* (Kijima *et al.* 1998). Likewise, the same behavior was obtained in the present work, when the cell-cycle of *C. ammoniagenes* was arrested using cephalexin (c.f. Results, Fig 3.1). This result may explain that the FtsI play the same role at the same sequence events as described by *E. coli*. Since the peptidoglycan lies outside of the cytoplasmic membrane (Sun and Margolin 1998; Weiss *et al.* 1999), the role of FtsI is almost certainly to catalyze the synthesis of septal-peptidoglycan within the periplasm (Fig 4.1). On the other hand, the role of the FtsI (PBP3) as the septal-peptidoglycan biosynthetic enzyme derives from the fact that *in vitro* purified forms of this protein may perform both transpeptidase and transglycosylase activities. The latter activity is required for completion of

septum formation by the aide of gene products of *fts Q, L, W, N, and K* (Ayala *et al.* 1994; stage IV in Fig 4.1).
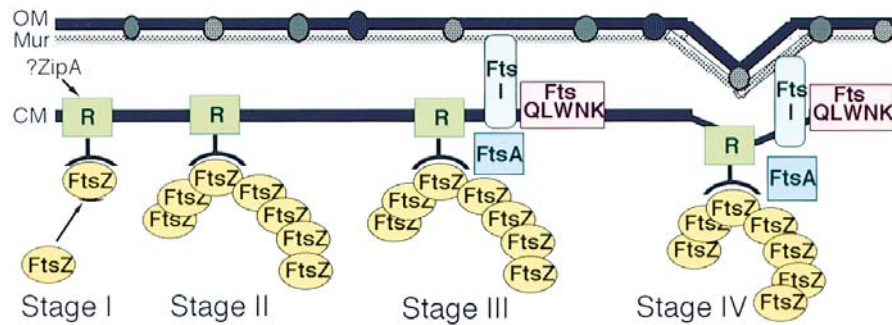
Fig 4.1 Process sequence of events during the *E. coli* division cycle (Rothfield and Justice 1997). OM,outer membrane; Mur, murein (peptidoglycan) layer of cell envelope; CM, cytoplasmic membrane; R, hypothetical FtsZ receptor.

Because of the inhibition of cell division by cephalexin which was carried out in nonsynchronized culture, the initiation of septum formation could be seen (Fig. 3.1) when cephalexin affected in the stage IV. However, initiation of septum formation was absent in other cells when the inhibition by cephalexin was at the stage III (Fig. 4.1), where the presence of only 25% of normal amount can be sufficient for initiation of septation (Ayala *et al.* 1994). In conclusion, the *ftsI* (*pbpB*) gene product, a penicillin-binding protein involved in the synthesis of septal peptidoglycan, is found to be essential in *C. ammoniagenes*. Moreover, it can be suggested that treatment with cephalexin, which is known as inhibitor for septation in *E. coli* (Spratt 1975) and *C. glutamicum* (Kijima *et al.* 1998), led to direct inhibition of the cell-cycle of *C. ammoniagenes* resulted in induction of only limited elongation.

A central and unresolved aspect of the cell-cycle in bacteria is the co-ordination between chromosome replication and cell division (Rothfield *et al.* 1999). For many years and largely from studies on *E. coli* (Donachie 2001), *Caulobacter crescentus* (MacAdams and Shapiro 2003) and *B. subtilis* (Harry *et al.* 1999), it was thought that this was likely to be accomplished by an obligatory link between the initiation of cell division and termination of a round of

replication (Boye and Nordström 2003). This conceals a complex regulatory network that co-ordinates the location and timing of variety of biochemical distinct processes. Clearly, it is known that the arrest of cell-cycle of *E. coli* can be achieved when its DNA replication is inhibited by drugs or mutations, as this has direct effects on chromosome segregation and indirect effects on cell division via the SOS response (Huisman *et al.* 1984). For example, novobiocin, an aminocoumarin antibiotic, blocks DNA replication by inhibiting the DNA-gyrase (Maxwell 1999). The main role of DNA gyrase in prokaryote are to introduce supercoiling of the chromosomal DNA. However, gyrase activity has an intrinsic relationship to several important biological functions. These functions include: DNA replication, transcription, and numerous other cellular functions besides supercoiling of chromosomal DNA. Replication and gyrase activity are closely linked functions (Woldringh and Odijk 1999). For instance, when antibiotics that specifically inhibit gyrase activity were used, inhibition of replication was also observed. This clearly illustrates the relatively coupled nature of these two functions. It is not surprising that gyrase activity would promote replication since negatively supercoiled DNA would probably promote DNA unwinding at the replication fork. The absence of periplasm invagination in the elongated cells (Fig. 3.3) indicates that arrest of cell division by inactivation of DNA gyrase occurs at an early period. In other words, coupling the DNA replication to DNA supercoiling may explains the early inhibition of cell-cycle in the C-period comparing with inhibition of septum formation which occurs in the D-period.

The inhibition of DNA precursor biosynthesis is the second indirect method for arresting the cell-cycle of *C. ammoniagenes* in the present work. The main role of increasing the cytoplasm (in the B-period) is to multiply all precursors requested for building the macromolecules in the further periods. Since the B-period is not seen at moderate and at high growth rates (cf. Introduction), the enzymes required for supplying with the primary metabolites may perform their roles in a pre-early stage of the C-period. The ribonucleotide reductase (RNR) is the only enzyme that catalyzes DNA precursor biosynthesis in all living cells (Stubbe and van der Donk 1998; Paesi-Toresan *et al.* 1998; Chabes and Thelander 2000, Follmann 2004). The Mn-RNR is expected to be controlled with respect to its

overall activity by the regulated stability of the limiting R2F protein as described for the Fe-RNR (Chabes and Thelander 2000). The RNR of *C. ammoniagenes* (Willing *et al.* 1988) and *C. glutamicum* (Abbouni 1999; Oehlmann and Auling 1999) has been reported to contain manganese (Mn-RNR) and consists of a large catalytic subunit R1E and a small radical bearing metallo-cofactor named R2F (Griepenburg *et al.* 1998). Quenching of the organic radical of the Mn-RNR, either by HU or MP, inhibits DNA precursor biosynthesis and generates pre-early versus regulated shut-down ('peak' behavior) of ribonucleotide reduction (Fig. 3.7). The pre-early shut-down behavior was proved by the similarity between activity of Mn-RNR inhibited with both studied radical scavengers, HU and MP.

The pre-early inhibition of the cell-cycle of the wild-type strain ATCC 6872 due to inactivation of Mn-RNR was confirmed by the complete absence of any morphological indications for starting septation (Fig. 3.5). Moreover, the elongated rods displayed after inhibition of DNA precursor biosynthesis due to shifting to the non-permissive temperature proved the pre-early arrest of cell-cycle of the ts-mutant CH31 (Fig. 3.8). The swelling and starting the formation of branched cells may indicate the increase (overproduction) of protein synthesis due to arrest of the cell-cycle of the CH31 strain.

In conclusion, it can be stated that all inhibition methods (either direct or indirect) used in the present work caused limited elongation in *C. ammoniagenes* (either wild-type or the mutant CH31)*,* but did not cause filamentation as in *E. coli*. Moreover, the swelling which accompanied the limited elongation when DNA replication was inhibited by gyrase indicates cell-cycle inhibition results in continued mass increase. In addition, branches seemed to occur at midcell to generate T-shape cells of the ts-mutant CH31 when DNA synthesis was inhibited by shifting the temperature to non-permissive conditions (Fig.3.8). The T-shaped cells were clearly formed when the morphological changes in the CH31 cells were studied under controlled conditions when growth was not permitted (Fig. 3.30). Breaches in peptidoglycan can occur at or near the division site in T-shaped cells resulting from the overexpression of certain proteins induced by the inhibition of RNR. In *Rhizobium meliloti*, the T-shaped cells were observed when its DNA synthesis was inhibited with mito-

mycin C or nalidixic acid (Latch and Margolin 1997). These researchers had demonstrated that the formation of T-shaped cells was resulted from FtsZ over-expression at the middle of the cell. On the other hand, the limited elongation observed by *C. ammoniagenes* and its mutant CH31 may suggests a unique mechanism of inhibition of the corynebacterial cell-cycle. This unique manner is similar to that was demonstrated in *C. glutamicum* (Kijima *et al.* 1998). Moreover, this cessation of cell elongation in *C. ammoniagenes* is caused not only by the direct inhibition of the cell-cycle but also by the indirect methods which were applied in this study.

4.1.2 Nucleotide accumulation due to inhibition of cell division

Corynebacterium ammoniagenes is used for the commercial production of different nucleotides which have wide applications in food industry (Han *et al.* 1997). Generally, microorganisms synthesize the metabolites necessary for their growth and sustenance making use of strong metabolic regulation and prevent-ing excessive accumulation. On other words, all cellular components increased exponentially with constant properties according to the growth requirements in this period. This behavior occurs only in that middle phase of the classic growth cycle and is known as balanced growth (Fig 4.2). The exponential synthesis is a property of the population and is independent of the synthetic pattern during the division cycle of the individual cell (Cooper 1991). By blocking DNA formation the increase of cell number is inhibited while the continuous exponential increase of the synthesis of biomass is remained. This state is known as unbalanced growth (for more details, see Fig. 4.2). This behavior is similar to that was described due to induction of unbalanced growth in *C. ammoniagenes* by manganese starvation (Auling *et al.* 1980).
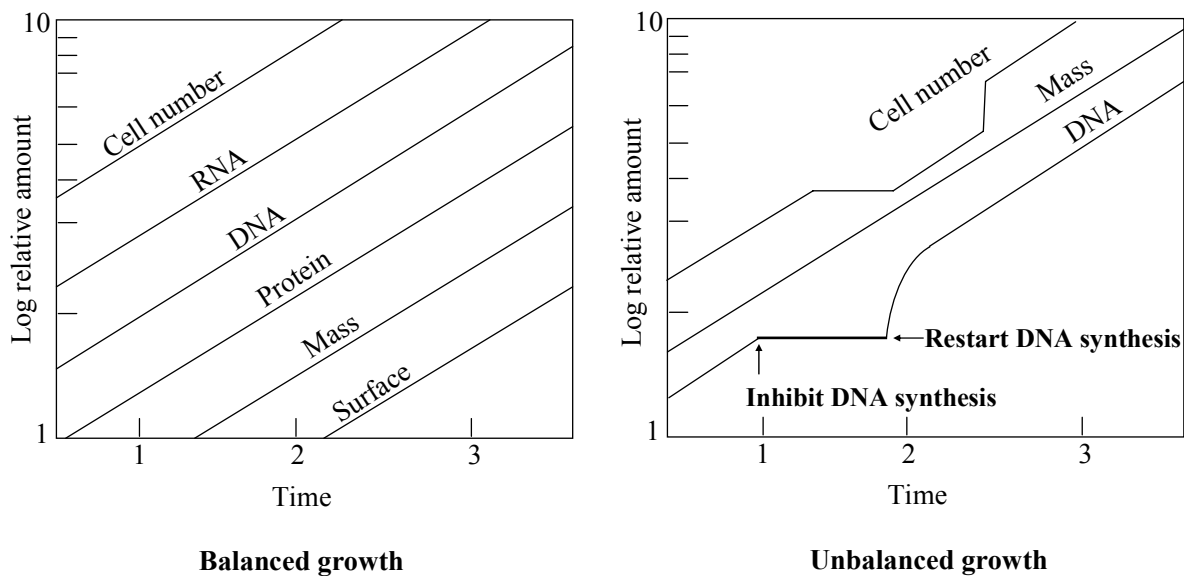
Fig. 4-2 Balanced and unbalanced growth (Cooper 1991). In the balanced growth all cell properties are increased exponentially, the average cell composition is constant. In the unbalanced growth, when DNA synthesis is inhibited for one interdivision time, then allowed resuming, the rate of DNA synthesis at the resumption of DNA replication is higher than the rate of DNA synthesis when inhibition started. Mass of cell continues exponentially during this period of DNA inhibition, whereas, cell number is inhibited after a short time.

The manganese ion is required and necessary not only for building the cell wall of *C. ammoniagenes* (Teshiba and Furuya 1989) but also for DNA precursor synthesis catalyzed by Mn-RNR (Auling *et al.* 1980). Therefore all experiments in the present work were carried out in the presence of excess Mn^{2+}. The continuous exponential increase of biomass synthesis even after the inhibition of DNA formation indicates the induction of unbalanced growth in *C. ammoniagenes* by the inactivation of DNA-gyrase with novobiocin (Sec.3.1.2) or Mn-RNR with radical scavengers (Sec. 3.1.3.1). Also, induction of unbalanced growth by the ts-mutant CH31 was achieved by shifting the temperature to the non-permissive conditions. Likewise, increasing of the biomass of the cultures inhibited by cephalexin (Sec. 3.1.1) indicates that the induction of unbalanced growth may occur by inhibiting septum formation even after DNA synthesis. This conclusion can be supported by those demonstrated in *Streptococcus sanguis* by Eisenberg (1973). The unbalanced growth of *S. sanguis* was characterized by gross morpho-

logical changes and continued mass increase after cell division had stopped. Abrupt cessation of RNA synthesis due to presence of oxygen induced the unbalanced growth of *S. sanguis*. This suggests that cell division was blocked at a stage which occurred after DNA synthesis, as described in this work when septum formation was inhibited.

The alteration of the pattern of nucleotide accumulation might offer some clues as to the nature of the biochemical lesion that caused the morphological changes in the inhibited cells. Because the production of primary metabolites is affected by the biomass of producer cells, the late stage of the logarithmic growth phase might be the suitable growth phase for blocking bacterial growth. This proper time of inhibition was determined in the present work as 7.5 h from the beginning of cultivation in the fermentation medium. On the other hand, at the level of the individual cell, pre-early inhibition of the cell-cycle was necessary for the overproduction of nucleotides by both the wild-type strain *C. ammoniagenes* ATCC 6872 and the ts-mutant CH31. This manner of blocking the cell-cycle by inactivation of the Mn-RNR which caused shortages of DNA precursors in rapidly growing cultures lead to nucleotide overproduction (Sec. 3.1.3). Likewise, the large accumulated amount of NAD^+ by the ts-mutant CH31 indicates the pre-early inhibition of the cell-cycle. Noticeably, the similarity between the behavior of accumulation of NAD^+ by the ts-mutant CH31 at 27°C (Fig. 3.9) and that of the parent strain (*C. ammoniagenes* ATCC 6872, Fig.3.6) may imply that the *de novo* biosynthetic rates of purine nucleotide did not change during mutagenic treatment in this mutant. In conclusion, the efficiency of nucleotide accumulation was clearly affected by the proper time for repression of the cell growth and individual cell-cycle events.

4.2 Identification of the putative point mutation in the *nrdE^{ts}* of strain CH31

Prior to discussion of the point mutation in the *nrdE^{ts}* of *C. ammoniagenes* strain CH31, it should be pointed out that all DNA templates used in the sequencing experiments were isolated from the chromosomal DNA. These DNA targets were amplified *in vivo* in *E. coli*; not as PCR product. Moreover both *nrdE^{ts}* and *nrdE^{+}* from the strains CH31 and wild-type ATCC 6872, respectively, were sequenced twice and in both strands (5´→3´ and 3´→5´). The available sequence of the *nrdE* gene from the wild-type strain *C. ammoniagenes* ATCC 6872 in our laboratory was incomplete (Oehlmann 1998). However, the outcome of the comparison of the *nrdE^{ts}* gene (Sec. 3.2.1) with the published *nrdE* sequence of *C. ammoniagenes* ATCC 6872 (GeneBank® [gi:3077613], Fieschi *et al.* 1998) was rather unexpected. According to this comparison four point mutations would be present in the mutated gene of CH31, the first one at nt 1301 and the other three would be clustered within a stretch of only 43 bp at nt 2021, 2032, and 2063. The latter three base pairs substitutions are difficult to accept from a statistical point of view because of the random mutagenesis protocol used in the production of this mutant (Luo *et al.* 1997).

There are two assumptions to explain the putative presence of more than one different bp in the *nrdE^{ts}* gene (Sec. 3.2.1) concluded from alignment with that of *nrdE^{+}* reported by Fieschi *et al.* (1998) for strain ATCC 6872. The first is the occurrence of repeated changes in the nucleotide sequence as a result from natural evolution of strain ATCC 6872 used in this study, which has been cultivated in the Institute of Microbiology, Hannover University, since about twenty years. The second assumption is the possibility of sequencing errors. Again, the sequencing diagrams shown in Fig. 4.3 indicate the high quality of sequencing results obtained here. The high resolution at the disputed nucleotide positions excludes base pair substitutions at nt 2021, 2032, and 2063. However, only one point mutation was identified at nt 1301 when the sequence of the *nrdE^{ts}* was compared with the sequence of the parent strain ATCC 6872 (obtained in this work).
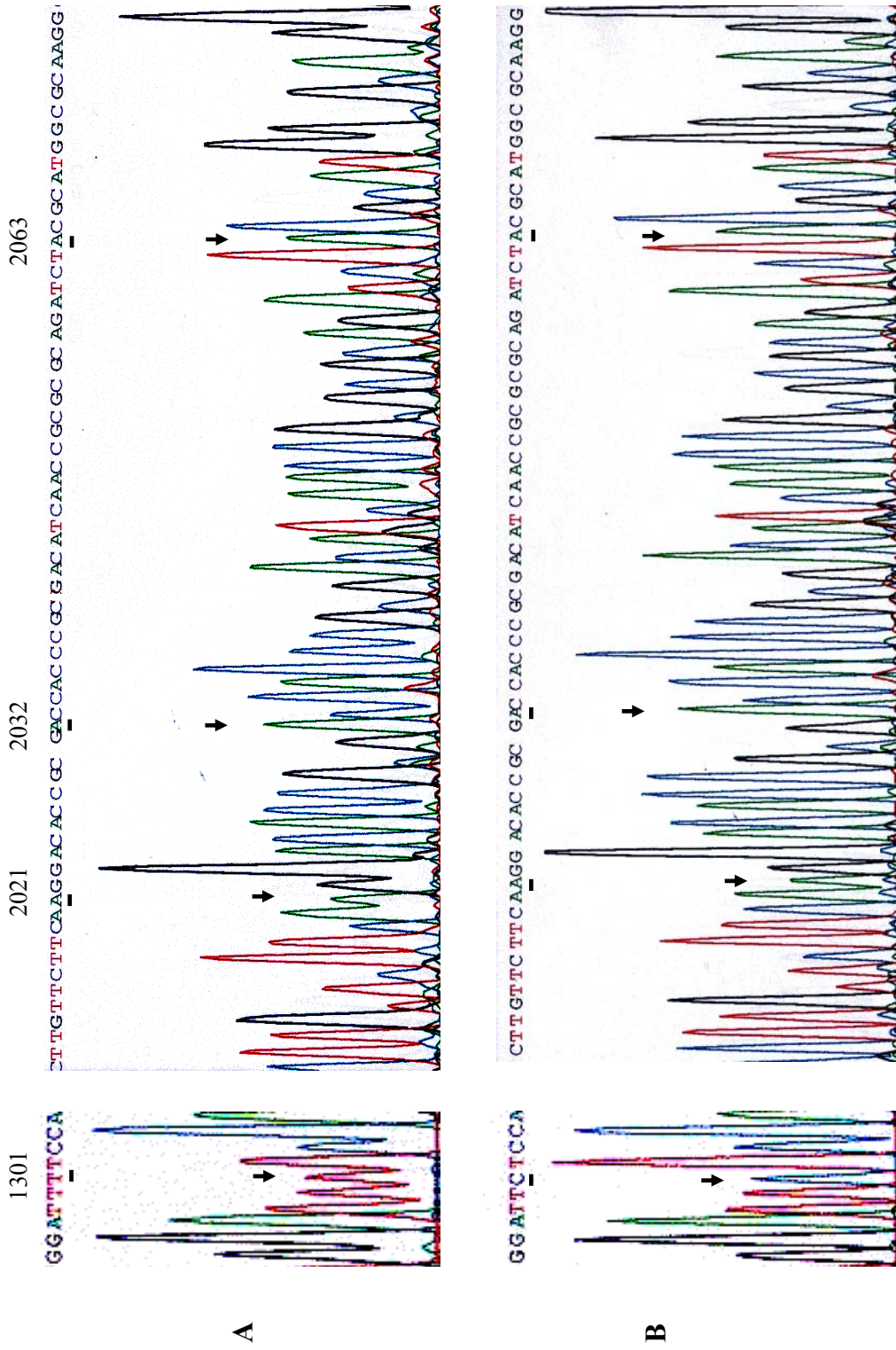
Fig. 4.3 Diagrams of partial sequences of the *nrd*^ts^ mutant CH31(A) and its parent strain *Corynebacterium ammoniagenes* ATCC 6872 (B). The bases at the positions 1301, 2021, 2032,and 2063 in the *nrdE* in both strains were indicated by arrows. Only the point mutation at the nt 1301 was approved.

Amino acid alignment with published amino acid sequences of the NrdE (also known as R1E) proteins from other species of *Corynebacterium* was done for further exclusion of clustered mutations in the *nrdE^ts* gene sequenced here. These sequences include two different sequences of the NrdE from the model organism of the corynebacteria, *Corynebacterium glutamicum* ATCC 13032, in addition to *C. diphtheriae* NCTC 13129, and *C. efficiens* Ys-314.

At the C-terminus (Fig. 4.4), extensive identity at the position A, B, and C was observed between the sequence of *nrdE* gene of the wild-type strain ATCC 6872 sequenced in this study and the nucleotide sequences of the other corynebacteria species. At these positions, the amino acids are lysine, threonine, and tyrosine, respectively, in the NrdE in all shown sequence of the *Corynebacterium* species except that of the ATCC 6872 published in GeneBank® [CAA70765, gi:3077613].

```
                              A    B              C
CG1-ATCC 13032   ATKYVDQGLSLTLFFKDTATTRDINRAQIYAWRKGIKTLYYIRLRQ   691

CG2-ATCC 13032   ATKYVDQGLSLTLFFKDTATTRDINRAQIYAWRKGIKTLYYIRLRQ   691

CE-Ys-314        ATKYVDQGLSLTLFFKDTATTRDINRAQIYAWRKGIKTLYYIRLRQ   704

CD-NCTC13129     ATKYVDQGLSLTLFFKDNVTTRDINRAQIYAWRKGIKSLYYIRLRQ   702

CA1-ATCC 6872    ATKYVDQGLSLTLFFRDTAATRDINRAQIFAWRKGIKTLYYIRLRQ   703

CA2-ATCC 6872    ATKYVDQGLSLTLFFKDTATTRDINRAQIYAWRKGIKTLYYIRLRQ   703
```

Fig.4.4 NrdE amino acids alignment from *C. glutamicum* ATCC 13032 (CG1) [NP_601730, gi:19553728] and (CG2) [AAD41036, gi:5230832]; *C. efficiens* (CE).[NP_739033, gi:23494266]; *C. ammoniagenes* ATCC 6872 (CA1) [CAA70765, gi:3077613] , (CA2) [the present work] and *C. diphtheriae* (CD).[NP_940204, gi:38234437].

The alignment analysis (Fig. 4.4) and the sequencing diagrams (Fig. 4.3) demonstrated that there is only one point mutation at the nt 1301 in the *nrdE* gene of the thermosensitive mutant CH31. This point mutation led to exhange the amino acid serine (Ser, or S) at the position 434 in the R1E to phenylalanine (Phe, or F).

The *nrdE* and *nrdF* genes encoding the R1E and R1F, respectively, are common among Gram-positive bacteria (Torrents *et al.* 2002). The ribonucleotide reductases of the R1E type lacks negative regulation by high concentration of dATP and is considered to belong to class Ib to distinguish it from the class I enzyme (Eliasson *et al.* 1996). This type of RNR enzymes was suggested to represent a new class, class VI Mn-RNR (Auling and Follmann 1994; Oehlmann and Auling 1999).
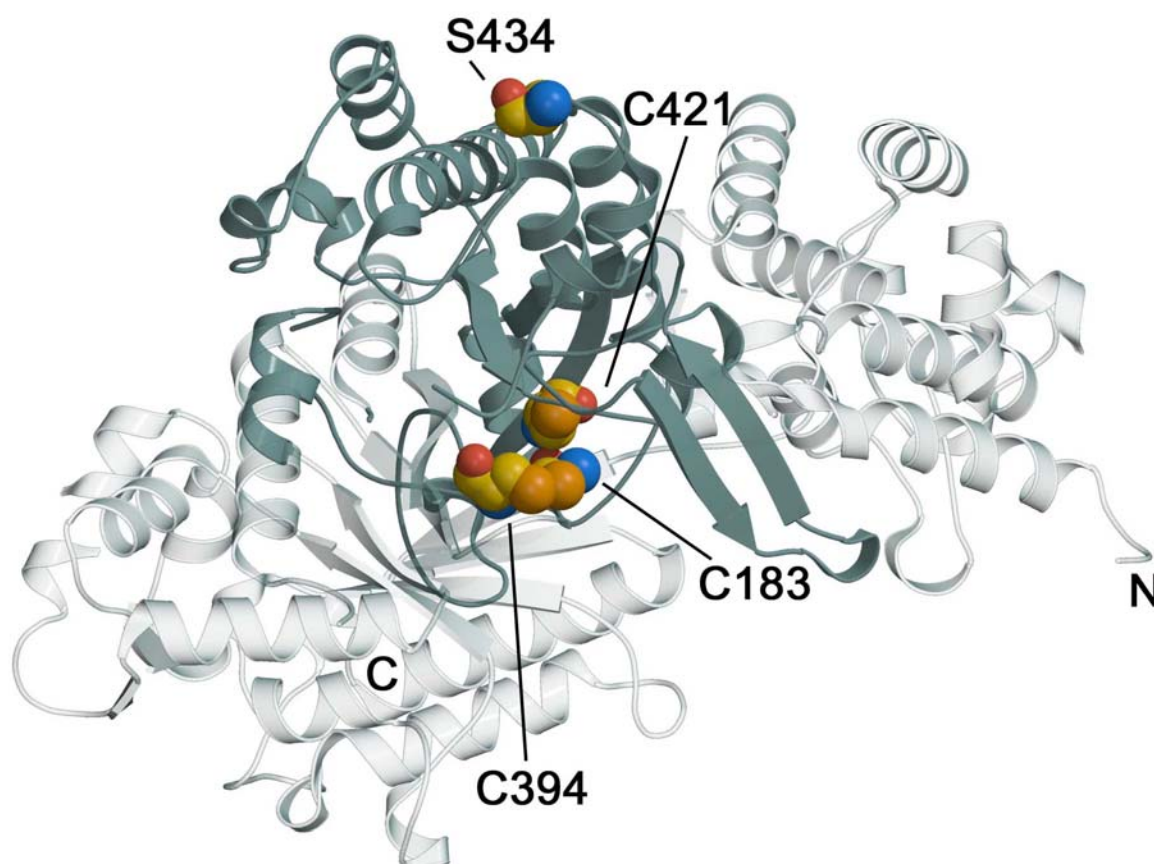
There are three cysteine (C) residues in the active site of the catalytic subunit R1E of *S. typhimurium*. The residue C388 in R1E corresponds to C439 in the R1 from *E. coli* (Uppsten *et al.* 2003). This residue is considered to be transiently transformed to a thiyl radical that removes a hydrogen atom from the 3′ carbon of the substrate initiating the catalytic transformation of the substrate to a 2′- deoxyribonucleotide (Stubbe *et al.* 2001). In the oxidized form, the redox-active cysteine residues 178 and 415 form a disulfide (Eriksson *et al.* 1998). The amino acid alignment analysis between the R1E of *C. ammoniagenes* ATCC 6872 (obtained in the present study) and that of *S. typhimurium* LT2 (Jordan *et al.* 1994; McClelland *et al.* 2001; Q08689) indicates 68.9% identity and 84% similarity (Fig. 4.5).

```
CA_ATCC 6872    VSCFLLRIEDNMESIGRAINSSLQLSKRGGGVALLLSNIRESGAPIKHIENQSSGIIPIM    240
St_LT2          VSCFLLRIEDNMESIGRAVNSALQLSKRGGGVAFLLSNLREAGAPIKRIENQSSGVIPVM    235
                **.***************:**:***********:****.**:*****:*******:**.*

CA_ATCC 6872    KMLEDAFSYANQLGARQGAGAVYLNAHHPDILNFLDTKRENADEKIRIKTLSLGIVIPDI    300
St_LT2          KMLEDAFSYANQLGARQGAGAVYLHAHHPDILRFLDTKRENADEKIRIKTLSLGVVIPDI    295
                ************************.*******.*********************:*****

CA_ATCC 6872    TFDLAKRNEDMYLFSPYDVERVYGKPFGDISVSDHYAEMVEDPRITKKKINARHFFQTVA    360
St_LT2          TFRLAKENAQMALFSPYDIQRRYGKPFGDIAISERYDELIADPHVRKTYINARDFFQTLA    355
                ** ***.* :* ******::* ********::*::* *:: **:: *. ****.****:*

CA_ATCC 6872    ELQFESGYPYIMFEDTVNNANPVKTGRINMSNLCSEILQVNSPSSFNDDLSYEEMGSDIS    420
St_LT2          EIQFESGYPYIMFEDTVNRANPIAG-RINMSNLCSEILQVNSASRYDDNLDYTHIGHDIS    414
                *:****************.***:   ****************.* ::*:*.* .:* ***

CA_ATCC 6872    CNLGSMNIALAMDSPDFGGTVEAAIRGLTAVADKTAIDSVPSIRHGNDQSHAIGLGQMNL    480
St_LT2          CNLGSLNIAHVMDSPDIGRTVETAIRGLTAVSDMSHIRSVPSIAAGNAASHAIGLGQMNL    474
                *****:*** .*****:* ***:*******:* : * *****  ** **********
```

Fig. 4.5 Sequence alignment of R1E from *C. ammoniagenes* ATCC 6872 (present work) and *S. typhimurium* LT2 (Q08689). Cysteine residues in the active site are highlighted (for more details see the text). The position of the point mutation in the region of the active site was indicated with arrow.

The residues C183, 394, and 421 in *C. ammoniagenes* ATCC 6872 corre-
spond to C178, 388, and 415 in *S. typhimurium*. According to this alignment
analysis, it may be suggested that the amino acid substitution S434F is located in
the active site region of the R1E of the ts-mutant CH31.

Moreover, the active site region of R1 contains a few additional conserved
residues such as serine, asparagine, and glycine (Eriksson *et al.* 1997). Likewise,
in corynebacteria, the serine residue at the active site is also conserved (Fig. 4.6).
Serine and threonine belong to the amino acids group with uncharged polar side-
chains which bear hydroxylic R groups of different size. These amino acids resi-
dues possess hydrogen bonds which are very important for interactions in folded
proteins by partial sharing of the hydrogen atom with other electronegative atoms.
The hydrogen atom that is shared has its electron delocalized onto the atom to
which it is covalently bonded, resulting in it possessing a partial positive charge.
These atoms are found on the polypeptide backbone and on side-chains of amino
acids. Unlike serine, phenyalanine is one of the aromatic amino acid residues
which participate in hydrophobic interactions.

```
                                      ↓
CG1-ATCC 13032      ISCNLGSLNVAMAMDSPNFEKTIETAIRGLTAVSEQTSIDSVPS     450
CG2-ATCC 13032      ISCNLGSLNVAMAMDSPNFEKTIETAIRGLTAVSEQTSIDSVPS     450
CE-Ys-314           ISCNLGSLNIAMAMDSPDFSRTIETAIRGLTAVSEQTSIDSVPS     463
CD-NCTC13129        ISCNLGSMNIAMAMDSTDFAKTVETAIRGLTAVSEQTSIDSVPS     261
CA1-ATCC 6872       ISCNLGSMNIALAMDSPDFGGTVEAAIRGLTAVADKTAIDSVPS     462
CA2-ATCC 6872       ISCNLGSMNIALAMDSPDFGGTVEAAIRGLTAVADKTAIDSVPS     462
                    *******:*:*:****.:*  *:*:**********:::*:******
```

Fig.4.6 Conserved serine residues at the putative active site region in NrdE from
different species of *Corynebacterium* genus. *C. glutamicum* ATCC 13032 (CG1)
[NP_601730, gi:19553728] and (CG2) [AAD41036, gi:5230832]; *C. efficiens*
(CE).[NP_739033, gi:23494266]; *C. ammoniagenes* ATCC 6872 (CA1) [CAA70765,
gi:3077613] , (CA2) [the present work] and *C. diphtheriae* (CD).[NP_940204,
gi:38234437]. The arrow indicates the position of amino acid substitution S434F in
the NrdE[ts] of the ts-mutant CH31.

Recently, the structure of the large subunit (R1E) of *Salmonella typhi-
murium* was identified by crystallization (Uppsten *et al.* 2003), however the co-
ordinates and structure factors will be released in Research Collaboratory for
structural Bioinformatics (RSCB) in May, 2004 (http://www.rcsb.org/pdb/).

Therefore, putative structure of the R1E of *C. ammoniagenes* was modeled based on the known structure of the R1 protein of *E. coli* (Fig. 4.7).

Fig. 4.7 Putative secondary structure (3PSSM model) of the R1E protein of *C. ammoniagenes*. Protein domain parser (PDP Molecule) was ribonucleotide reductase R1 protein of *Escherichia coli*. Side chains, where applicable, have been modeled by SCWRL program (Kelley *et al.* 2000). The figure was produced with Molscript (Kraulis, 1991) and rendered with Raster3D (Merritt und Bacon, 1997). N = N-terminus; C = C-terminus; Dark-part of the model with no gaps; Light-part of the model with gaps.[1]

Since the native structure of a protein depends only on its amino acid sequence, it should be possible, in principle, to predict the structure of a protein based only on its chemical and physical properties (e.g., the hydrophobicity, size,

[1] This model was made with the aid of Dr. Matthias Stehr, Department of Genomic Research, German Research Centre for Biotechnology.

hydrogen-bonding property, and charge of each of its amino acid residues (Voet *et al.* 1999).

In the putative secondary structure of the R1E protein of *C. ammoniagenes* (Fig 4.7) the serine residue (S434) is located at the surface of the molecule. Therefore, it can be suggested that the substitution of the polar residue serine with the hydrophobic residue phenylalanine makes the R1E protein less stable. This instability is responsible for altered phenotype of the ts-mutant CH31 at the non-permissive temperature.

Dramatic effects due to conversion of serine to phenylalanine or another hydrophobic residue in other organisms were stated in the recent literature. For example, introduction of a point mutation by exchanging serine to phenylalanine in the GyrA protein of *Staphylococcus intermedius* led to a gyrase which is no longer susceptible to inhibition by the antibiotic nalidixic acid, presumably due to conformational changes in the protein structure (Lioyd *et al.* 1999). Moreover, the substitution of a serine residue to valine in the active site of the flavanone 3b-hydroxylase of *Petunia hybrida* led to loss 99% of the enzyme activity (Lukacin *et al.* 2000).

4.3 Genetic complementation of the ts-mutant CH31 with the *nrdE*$^+$ gene

Although the amino acid production of *Corynebacterium glutamicum* has been known since the 1950s, the first reports on the genetic engineering of *C. glutamicum* and its close relative bacteria, only appeared in 1984 (Miwa *et al.* 1984; Santamaria *et al.* 1984). Since then, the repertoires of genetic engineering methods as well as the understanding of the biochemical pathways and genetic systems in these organisms have increased significantly (Pühler 1993). The complete sequences of several species of corynebacteria are available in GeneBank$^®$ database and started a new era of research with these organisms (Nishio *et al.* 2003; Tauch *et al.* 2003). Essential prerequisites for the manipulation of bacteria of any kind are the availability of vectors derived from plasmids or bacteriophages and efficient DNA transfer systems. Different systems for introducing DNA into corynebacteria have been established. These include fusion of protoplast, transformation

of spheroplasts and protoplasts, transfection of protoplasts, transduction, conjugation, and electroporation. Of these methods, the electroporation is the fast and most widely used. Considerable progress has been made during the last years in understanding the genetic organization and molecular biology of plasmids from *C. glutamicum*. This progress was mainly achieved by systematic sequencing studies, which resulted in the determination of a number of complete plasmid sequences (Ohnishi *et al.* 2002; Nakata *et al.* 2003). The *C. glutamicum/E. coli* shuttle vector pXMJ19 is one of the pBL1 family of *C. glutamicum* plasmids (Jakoby *et al.* 1999). This shuttle vector was constructed on the basis of the high copy number *E.coli* plasmid pK18 and the cryptic low copy number *C. glutamicum* plasmid pBL1. In the present work, the vector pXMJ19 was applied for overexpression of the $nrdE^+$ gene of *C. ammoniagenes* ATCC 6872 after introduction in the ts-mutant CH31.

The successful overexpression of the wild-type gene $nrdE^+$, carried in the plasmid pXE6872, in the ts-mutant CH31 was revealed by growth of complemented cells at both permissive (27°C) and restrictive (37°C) temperatures (Fig. 3.22) However, because of the *nrdE* is an essential enzyme in all living cells, the pXE6872 could not be expressed in the ts-mutant CH31 at 37°C without pre-induction with IPTG at 27°C. This induction period guarantee production of $NrdE^+$ in the complemented cells sufficient to support their growth at the restrictive temperature. Under the same conditions, absence of the growth of the ts-mutant CH31/pXECH31, carried the $nrdE^{ts}$ gene, at 37°C indicate clearly that the point mutation which was detected in this study is the only reason responsible for thermosensitivity of the mutant CH31. Moreover, the capability of the CH31/pXE6872 cells to grow at 37°C without observation of elongation or swelling verifies the overproduction of $NrdE^+$ morphologically (Fig.3.23). On the other hand, the overproduction of either the $NrdE^+$ or $NrdE^{ts}$ in the ts-mutant CH31, which was indicated by immunoblotting (Fig 3.21), demonstrates the ability of gene expression in *Corynebacteium* under control by induction with IPTG.

4.4   Exploitation of the ts-mutant CH31 for nucleotide accumulation

Bioprocesses to produce nucleotides have been classified into two types in general. One is the direct bioprocess accumulation of 5´-IMP and 5´-XMP by *Corynebacterium ammoniagenes*. The second is a two-step process composed of production of nucleosides by bioprocess followed by enzymatic phosphorylation. The second production process was developed recently by compination of the strong ATP-regenerating activity of *C. ammoniagenes* with a reaction catalyzed by other microbial cells (Beppu 2000). Compared to the direct bioprocess, the hybrid system allowed far higher productivity. In the latter method the IMP was produced from inosine which was accumulated by an adenine and guanine auxotrophic mutant strain of *C. ammoniagenes* (Mori *et al.* 1997).

In the present work, an alternative bioprocess for single-step accumulation of 5´-IMP using the ts-mutant CH31 was described. This process seems to have several advantages:

- Accumulation of 5´-IMP can be achieved by shifting the temperature without addition of any other chemicals. This procedure guarantees inhibition of only the RNR in contrast to using other inhibitors, which may affect other systems or reactions in the inhibited cell.
- The ts-mutant CH31 has a capability to accumulate 5´-IMP in the fermentation broth in the presence of high Mn^{2+} ion concentrations which interfer with nucleotide accumulation by *C. ammoniagenes* ATCC 6872 (Oka *et al.* 1968; Auling *et al.* 1980; cf. INTRODUCTION).
- The 5´-IMP productivity can be improved using the hypoxanthine as precursor for accumulation of 5´-IMP through salvage pathway, discussed below.

Nucleotides are produced either by *de novo* synthesis or salvage biosynthetic pathways. Synthesis of nucleotides from purine bases and purine nucleosides takes place in a series of steps known as salvage pathways. NH_3 from deamination of purine bases and formation of pentose phosphates from cleavage of nucleosides may serve as nitrogen and carbon sources, respectively, in

cellular metabolism (Switzer *et al.* 2002). Genes encoding functions involved in purine transport, salvage, and interconversion are more available after the completion of genomic sequencing of several microorganisms including corynebacteria (see below). The free purine bases such as adenine, guanine, and hypoxanthine may be reconverted to their corresponding nucleotides by phosphoribosylation. Two key transferase enzymes are involved in the salvage of purines: adenosine phosphoribosyltransferase (APRT), which catalyzes the following reaction:

$$\text{adenine} + \text{PRPP} \longrightarrow \text{AMP} + \text{PP}_i$$
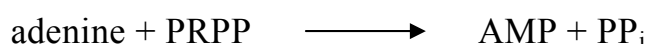
and hypoxanthine-guanine phosphoribosyltransferase (HGPRT), which catalyzes the following reactions:

$$\text{hypoxanthine} + \text{PRPP} \longrightarrow \text{IMP} + \text{PP}_i$$
$$\text{guanine} + \text{PRPP} \longrightarrow \text{GMP} + \text{PP}_i$$
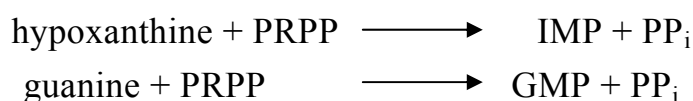
In addition, adenine and guanine compounds are interconvertible through the intermediate formation of IMP. The key reactions are the formation of IMP from GMP catalyzed by GMP reductase and conversion of adenine to IMP via hypoxanthine catalyzed by adenine deaminase and HGPRT. IMP may also be formed from ATP via the intermediate formation of aminoimidazolecarboxamide ribotide (AICAR), which is a byproduct of histidine biosynthesis (Switzer *et al.* 2002).

The genomic DNA of the amino acid producers *Corynebacterium glutamicum* (Wehmeier *et al.* 1998) and *C. efficiens* (Nishio *et al.* 2003) were completely sequenced. The three salvage enzymes HGPRT, APRT, and GMP-reductase were identified in both *C. efficiens* (Nishio *et al.* 2003; Q8FPL0) and *C. glutamicum* (Wehmeier *et al.* 1998; BAC00073). The salvage mechanism of conversion of hypoxanthine to IMP catalyzed by the key enzyme HGPRT is presented in Fig. 4.8.
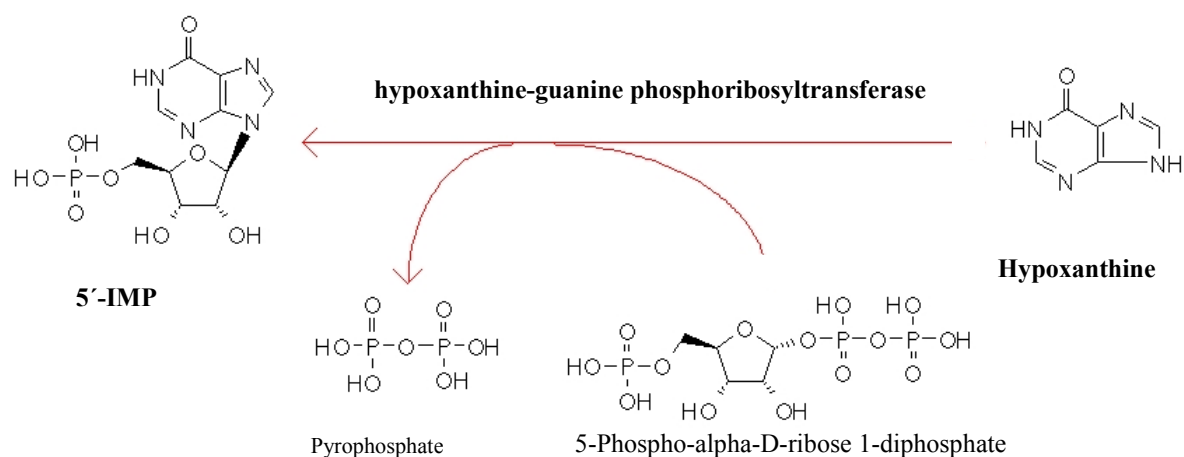
Fig. 4.8 IMP-salvage biosynthesis formed by catalytic activity of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) in both *C. glutamicum* and *C. efficiens* when hypoxanthine was used as precursor (http://www.genome.ad.jp/dbget-bin/www_bget?rn+R00190%2BR01132%2BR01229%2BR02142)

In the present study hypoxanthine was added to the mineral medium simultaneously with shifting the temperature to 37°C to enhance the accumulation of IMP by the ts-mutant CH31. The resulting dramatic increase (tenfold) in the accumulation of IMP indicates the successful conversion of the added hypoxanthine through salvage nucleotide mechanism. This can be explained by an increase in the expression of nucleotide salvage pathway genes as observed in *E. coli* when it was grown in minimal medium (Tao *et al.* 1999). In the relatives of corynebacteria, salvage biosynthesis was exploited for the production of adenosine 3´, 5´-monophosphate (cAMP) through sequential mutational improvement of *Microbacterium sp.* (Ishiyama 1990). Overproduction of the NAD^+ by a salvage biosynthetic pathway using *Corynebacterium ammoniagenes* and a high-salt mineral medium was first shown by Nakayama *et al.* (1968). A putative biosynthetic scheme for *de novo* and salvage mechanisms in *Corynebacterium ammoniagenes* (formerly, *Brevibacterium ammoniagenes*) was discussed based on biochemical data (Viereck 1975). Recently, the genome net database service ´´Kyoto Encyclopedia of Genes and Genomes´´ designs metabolic maps based on the combination of putative and identified genes.

(http://www.genome.ad.jp/kegg/).

The metabolic map of NAD^+ metabolism in prokaryotes as a model for nucleotide accumulation is shown in Fig. 4.9. NAD^+ is synthesized in bacteria by a complex network of *de novo* and salvage pathways (Kurnasov *et al.* 2002). Five enzymes are involved in *de novo* biosynthesis of NAD^+ from aspartate, aspartate oxidase [EC:1.4.3.16], dihydroxyacetone phosphate quinolinate synthase [NadA], quinolinate phosphoribosyltransferase [EC:2.4.2.19], nicotinic acid mononucleotide adenylyltransferase [EC:2.7.7.18], and NAD synthetase [EC:6.3.5.1] (Switzer *et al.* 2002).

As the rate-limiting enzyme, catalyzing the first reaction in NAD salvage synthesis, nicotinate phosphoribosyltransferase (NAPRTase, EC:2.4.2.11) is of important interest for studies of intracellular pyridine nucleotide pool regulation (Fig.4.9). This enzyme was identified in *Corynebacterium ammoniagenes* ATCC 6872 by Dulyaninova *et al.* (2000). The enzyme NAPRTase catalyzes the conversion of nicotinate to nicotinate D-ribonucleotide through salvage pathway (Fig. 4.9). Nicotinic acid mononucleotide adenyltransferase [EC:2.7.7.18] was also suggested in other bacteria as one of the salvage enzymes (Kurnasov *et al.* 2002). Due to addition of nicotinate and adenine as precursors and the data in the present work it is suggested that both salvage enzymes were used by the *C. ammoniagenes* wild-type and the ts-mutant for accumulation of NAD^+.

Fig. 4.9 Nicotinate and nicotinamide metabolism - Reference pathway (http://www.genome.ad.jp/dbgetbin/getpathway? orgname=map&mapno=00760). NAD$^+$ biosynthesis by *de novo* (light-gray background) and salvage (dark-gray background) pathways were discussed (for more details see the text).

Outlook

After completion the characterization of the ts-mutant CH31 (Luo *et al.* 1997; this work), the amino acid substitution S434F detected here in NrdE^{ts} is suggested as the only reason responsible for the thermosensitivity of this mutant. According to this result, further work can be suggested:

- To understand the ability of the ts-mutant CH31 to grow at permissive or non-permissive temperatures and to produce nucleotides after inhibition of its growth, it is critical to unravel the regulatory circuits that control its cell cycle. In particular, the bacterial cells have to control DNA replication, chromosome segregation, and cytokinesis temporally and spatially. These events are coordinated with growth. The periodicity of these cell cycle events is accompanied by oscillations of gene expression. Thus, studying cell cycle-dependent gene expression in a global manner not only catalogues periodically expressed genes but can also help to identify genes with novel cell cycle functions. Microarray transcription studies are suggested to characterize gene expression through the cell-cycle of the ts-mutant CH31 under conditions of direct and indirect inhibition. Also, the cell-cycle transcription factors for an important cluster of genes could be identified. For example, the transcriptional regulator CtrA controls several key cell-cycle events in *Caulobacter crescentus*, including the initiation of DNA replication, DNA methylation, and cell division. Certain promoter (P1) which control expression of *ctrA* is activated after inhibition of DNA replication in the early predivisional cell (Domian *et al.* 1999).

- The approach of the successful expression of the *nrdE* genes in the ts-mutant CH31 described in this work could be extended to study and characterize the ribonucleotide reductase from the related microorganisms especially of pathogenic organisms, such as *Mycobacterium tuberculosis*.

- In the field of nucleotide production the characterized ts-mutant CH31 could be developed by introducing further mutations in order to increase its

ability for accumulation of nucleotides. One of the most effective mutations for increasing the yield of nucleotide production is to release the control system of feedback inhibition and feedback repression. Auxotrophic mutants such as, adenine and guanine leaky mutants could be applied to overcome this regulation in order to increase the IMP production.

5   SUMMARY

The ts-mutant CH31 was generated from *C. ammoniagenes* ATCC 6872 by random mutagenesis (using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine) and biochemically characterized as a *nrd*^{ts} (*n*ucleotide *red*uction *t*hermo*s*ensitive) mutant (Luo *et al.* 1997). According to this biochemical characterization, the putative point mutation was located in the *nrdE* gene, encoding the large subunit of ribonucleotide reductase (RNR). To identify this point mutation the 5.2 kb *Xma*I-fragment containing the *nrdE* gene was isolated from the chromosomal DNA of the ts-mutant CH31 or its parent strain ATCC 6872. These fragments were cloned in pUC18 plasmid and transformed in *E. coli*. The *nrdE*$^{+}$ from the wild-type strain ATCC 6872 and the *nrdE*^{ts} from the ts-mutant CH31 were sequenced after *in vivo* amplification in *E. coli*. The nucleotide alignment of the *nrdE* genes reveled a nucleotide substitution at position 1301 from cytosine to thymine. This base pair substitution led to an exhange in the amino acid sequence of the mutant NrdE (R1E) protein at the position 434 of serine (S) with phenylalanine (F). After comparing the sequence obtained from the R1E of *C. ammoniagenes* with the R1E from *Salmonella typhimurium* (Uppsten *et al.* 2003), it can be demonstrated that the S434F mutation is located in a region flanking the active site of the R1E protein of strain CH31. To determine the responsibility of the S434F mutation for the thermosensitivity of the ts-mutant CH31, the complete sequences of *nrdE*$^{+}$ or *nrdE*^{ts} were cloned, without any flanking nucleotides, in the *C. glutamicum/E. coli* shuttle vector pXMJ19. The constructed plasmids were introduced and expressed in the ts-mutant CH31 by induction with 1mM IPTG. The ts-mutant CH31 complemented with the *nrdE*$^{+}$ restored its phenotype and grew normally at the non-permissive temperature without displaying any morphological changes. From the results obtained it can be concluded that the thermosensitive mutant CH31 has only a single amino acid substitution S434F localized in its R1E.

Microorganisms synthesize the metabolites necessary for their growth and sustenance making use of strong metabolic regulation and preventing excessive accumulation. In the present work, overproduction of the metabolites by *Corynebacterium ammoniagenes* can be achieved by arresting of its cell-cycle.

Therefore, the correlation between the nucleotide accumulation (NAD$^+$ as a model for nucleotide fermentation) and arrest of the cell-cycle was studied. The cell-cycle of the wild-type strain *C. ammoniagenes* was inhibited directly or indirectly. Cephalexin, an inhibitor of septum peptidoglycan synthetase (FtsI), was used for direct inhibition of the cell-cycle. Indirect inhibition of the cell-cycle was achieved by inhibition of DNA-gyrase by novobiocin or ribonucleotide reductase (RNR) by radical scavengers. Both direct and indirect inhibition of the cell-cycle of *C. ammoniagenes* ATCC 6872 led to induction of only limited cell elongation, 2-3 times the length of the normal corynebacterial rods. Likewise, the cell-cycle of the ts-mutant CH31 was indirectly inhibited by inactivation of its RNR due to shifting the temperature to the non-permissive temperature (37°C). The highest accumulated NAD$^+$ (1.5 g / l) was obtained by the strain CH31. Furthermore, the ts-mutant CH31 was utilized in an alternative biological process for the production of IMP. Enhancement of nucleotide accumulation was achieved by addition of precursors for exploitation of the salvage pathway. Production of IMP (4.3 g / l) in a 10-liter bioreactor was successfully performed. The metabolic pathway of NAD$^+$ synthesis as a model for nucleotide biosynthesis was discussed.

6 REFERENCES

Abbouni B (1999) Biochemische Charakterisierung der Ribonucleotid-Reduktase aus *Cory-nebacterium glutamicum* im Vergleich zum Manganenzym aus *Corynebacterium ammoniagenes*. Ph.D. thesis, Hannover University, Germany.

Abbouni B, Elhariry HM, Auling G (2003) Arrest of cell cycle by inhibition of ribonucleotide reductase induces accumulation of NAD^+ by Mn^{2+} supplemented growth of *Corynebacterium ammoniagenes*. Biotechnol. Lett. 25: 143-147.

Anderson TM (2000) Industrial fermentation process. *In*: Lederberg J (ed.) Encyclopedia of microbiology, 2nd edn. Academic Press, New York, pp 767-781.

Asano Y, Mihara Y, Yamada H (1999) A new enzymatic method of selective phosphoryla-tion of nucleotides. J. Mol. Catal. B. Enzymatic 6: 271-277.

Auling G (1980) Nucleotidstoffwechsel in *Brevibacterium ammoniagenes* und *Micrococcus sodonensis*. Habilitationsschrift, Univ. Hannover.

Auling G, Follmann H (1994) Manganese-dependent ribonucleotide reduction and over-production of nucleotides in coryneform bacteria. *In*: Siegel H, Siegel A (eds.) Metal ions in biological systems. Marcel Dekker, Inc, N.Y., Basel, Hong Kong, pp 131-161.

Auling G, Thaler M, Diekmann H (1980) Parameters of unbalanced growth and reversible inhibition of deoxyribonucleic acid synthesis in *Brevibacterium ammoniagenes* ATCC 6872 induced by depletion of Mn^{2+}. Inhibitor studies on the reversibility of deoxyribonucleic acid synthesis. Arch. Microbiol. 127: 105-114.

Ausubel FA, Brent R, Kingston RE, Moor DD, Seidemann JG, Smitz JA, Struhl K (1990) Current protocols in molecular biology. Creene Publishing and Wiley Inc. New York, pp 77-93.

Ayala JA, Garrido T, De Pedro MA, Vicente M (1994) Molecular biology of bacterial septation. *In*: Ghuysen JM, Hakenbeck R (eds.) Bacterial cell wall. Elsevier Sci. BV, pp 73-101

Beall B, Lutkenhaus J (1991). FtsZ in *Bacillus subtilis* is required for vegetative septation and for asymmetric septation during sporulation. Genes Dev. 5: 447-455.

Beppu T (2000) Development of applied microbiology to modern biotechnology in Japan. *In*: Scheper T (ed.) Advances in biochemical engineering and biotechnology, Vol. 59. Springer-Verlag, Berlin Heidelberg. pp 42-70.

Beuse M (1998) Populationsverteilung und Wachstumsmodi bei *Saccharomyces cerevisiae* in synchronen Chemostatkulturen. Ph.D. thesis, Hannover University, Germany.

Birnboim H, Doly J (1979) Rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 9: 2989-2998.

Bott M (2001) Personal communication, Institute of Biotechnology 1 Forschungszentrum, Jülich GmbH Jülich, Germany.

Boye E, Nordström K (2003) Coupling the cell cycle to growth: A look at the parameters that regulate cell-cycle events. EMBO reports. 4: 757-760.

Cha JH, Stewart GC (1997) The *divIVA* minicell locus of *Bacillus subtilis*. J. Bacteriol. 179: 1671–1683.

Chabes A, Thelander L (2000) Controlled protein degradation regulates ribonucleotide reductase activity in proliferating mammalian cells during the normal cell cycle and in response to DNA damage and replication blocks. J. Biol. Chem. 275:17747-17753.

Cheetham PSJ (1993) The use of biotransformation for the production of flavours and fragrances. Trends Biotechnol. 11: 478-488.

Ciotti MM, Kaplan NO (1957) Procedures for determination of pyrimidine nucleotides. *In*: Colowick SP, Kaplan NO (eds.). Meth. Enzym. 3: 891-892. Academic Press, N Y, London.

Cooper S (1991) Bacterial growth and cell division. Academic Press, N.Y.

Dai K, Lutkenhaus J (1991). *ftsZ* is an essential cell division gene in *Escherichia coli*. J. Bacteriol. 173: 3500-3506.

Deibler KD, Delwiche J (2004) Handbook of flavor characterization; sensory analysis, chemistry, and physiology. Marcel Dekker, Inc. New York.

Demain AL (2000) Microbial biotechnology. TIBTECH 18: 26-31

Domian IJ, Reisenauer A, Shapiro AL (1999) Feedback control of a master bacterial cell-cycle regulator. Proc. Natl. Acad. Sci. USA. 96: 6648-6653.

Donachie WD (2001) Co-ordinate regulation of the *Escherichia coli* cell cycle or the cloud of unknowing. Mol. Microbiol. 40 (4): 779-785.

Dulyaninova NG, Podlepa EM, Toulokhonova1 LV, Bykhovsky VY (2000) Salvage

pathway for NAD biosynthesis in *Brevibacterium ammoniagenes*: regulatory properties of triphosphate-dependent nicotinate phosphoribosyl-transferase. Biochim. Biophys. Acta 1478: 211-220.

Edwards DH, Errington J (1997) The *Bacillus subtilis* DivIVA protein targets to the division septum and controls the site specificity of cell division. Mol. Microbiol. 24: 905-915.

Eisenberg RJ (1973) Induction of unbalanced growth and death of *Streptococcus sanguis* by oxygen. J. Bacteriol. 116: 183-191.

Eliasson R, Pontis E, Jordan A, Reichard P (1996) Allosteric regulation of the third ribonucleotide reductase (NrdEF enzyme) from Enterobacteriaceae. J. Biol. Chem. 271: 26582-26587.

Eriksson M, Jordan A, Eklund H (1998) Structure of *Salmonella typhimurium nrdF* ribonucleotide reductase in its oxidized and reduced form. Biochemistry. 37: 13359-13369.

Eriksson M, Uhlin U, Ramaswamy S, Ekberg M, Regnström K, Sjöberg BM, Eklund H (1997) Binding of substrate and allosteric effectors to ribonucleotide reductase protein R1: Reduction of active site cysteines promotes substrate binding. Structure 5: 1077-1092.

Fieschi F, Torrents E, Toulokhonova L, Jordan A, Hellman U, Barbe J, Gibert I, Karlsson M, Sjöberg B (1998) The manganese-containing ribonucleotide reductase of *Corynebacterium ammoniagenes* is a class Ib enzyme. J. Biol. Chem. 273: 4329-4337.

Follmann H (2004) Deoxyribonucleotides: the unusual chemistry and biochemistry of DNA precursors. Chem. Soc. Rev. 33: 225-233.

Fudou R, Jojima Y, Seto A, Yamada K, Kimura E, Nakamatsu T, Hiraishi A, Yamanaka S (2002) *Corynebacterium efficiens* sp. *nov.*, a glutamic-acid-producing species from soil and vegetables. Int. J. Syst. Evol. Microbiol. 52: 1127-1131.

Gellert M, O'Dea MH, Itoh T, Tomizawa J (1976) Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc Natl Acad Sci U S A. 73: 4474-8447.

Glick BR, Pasternak JJ (2003) Molecular biotechnology: Principles and applications for recombinant DNA. ASM Press, Washington, D.C., pp 3-13.

González CF, Gil JA, Mateos LM, Schwarzer A, Schäfer A, Kalinowski J, Pühler A, Martin JF (1996) Construction of L-lysine-overproducing strains by targeted disruption of

the *hom* and *thrB* genes. Appl. Microbiol. Biotechnol. 46: 554-558.

Gordon GS, Sitnikov D, Webb CD, Teleman A, Straight A, Losick R, Murray AW, Wright A (1997) Chromosome and low copy plasmid segregation in *E. coli*: Visual evidence for distinct mechanisms. Cell 90: 1113-1121

Griepenburg U, Kappl R, Hüttermann J, Auling G (1998) A divalent metal site in the small subunit of the manganese-dependent ribonucleotide reductase of *Corynebacterium ammoniagenes*. Biochem. 37: 7992-7996.

Griepenburg U, Laßmann G, Auling G (1996) Detection of a stable free radical in the B2 subunit of the manganese-dependent ribonucleotide reductase (Mn-RNR) of *Corynebacterium ammoniagenes*. Free Rad. Res. 261: 473-481.

Han JK, Chung SO, Lee JH, Byun SM (1997) 6´-Mercaptoguanosine-resistance is related with *purF* gene encoding 5´-phosphoribosyl-1´-pyrophosphate amidotransferase in inosine-5´-monophosphate overproducing *Brevibacterium ammoniagenes*. Biotechnol. Lett. 19: 79-83.

Harry EJ, Rodwell J, Wake RG (1999) Co-ordinating DNA replication with cell division in bacteria: a link between the early stages of a round of replication and mid-cell Z-ring assembly. Mol. Microbiol. 33: 33-40.

Hawker R, Niday E, Gordon J (1982) A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. 119: 142-147.

Honrubia MP, Fernández FJ, Gil JA (1998) Identification, characterization, and chromosomal organization of the *ftsZ* gene from *Brevibacterium lactofermentum*. Mol. Gen. Genet. 259: 97-104.

Huisman O, D'Ari R, Gottesman S (1984) Cell-division control in *Escherichia coli*: specific induction of the SOS function SfiA protein is sufficient to block septation. Proc Natl Acad Sci USA. 81: 4490-4494.

Ishiyama J (1990) Isolation of mutants with improved production of cAMP from *Microbacterium sp* no. 205 (ATCC 21376). Appl. Microbiol. Biotechnol. 34: 359-63.

Jacobs C, Shapiro L (1999) Bacterial cell division: A moveable feast. Proc. Natl. Acad. Sci. USA. 96: 5891-5893.

Jakoby M, Ngouoto-Nkii CE, Burkovski A (1999) Construction and application of new *Corynebacterium glutamicum* vectors. Biotechnol. Tech. 13: 437-441.

Jensen RB, Wang SC, Shapiro L (2001) A moving DNA replication factory in *Caulobacter crescentus*. EMBO J. 20: 4952-4963.

Jordan A, Gibert I, Barbe J (1994) Cloning and sequencing of the genes from *Salmonella typhimurium* encoding a new bacterial ribonucleotide reductase. J. Bacteriol. 176: 3420-3427.

Kawamura Y, Kare MR (1987) Umami: A basic taste, physiology, Biochemistry, nutrition, and food science. Marcel Dekker, Inc. New York.

Kelley LA, MacCallum RM, Sternberg MJE (2000) Enhanced genome annotation using structural profiles in the program 3D-PSSM. J. Mol. Biol. 299: 499-520.

Kijima N, Goyal D, Takada A, Wachi M, Nagai K (1998) Induction of only limited elongation instead of filamentation by inhibition of cell division in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 50: 227-232.

Kobayashi M, Asia Y, Hatakeyama K, Kijima N, Wachi M, Nagai K, Yukawa H (1997) Cloning, sequencing, and characterization of the *ftsZ* gene from coryneform bacteria. Biochem. Biophysic. Res. Commun. 236: 383-388.

Koch AL (2001) Bacterial growth and form. Kluwer Academic Publisher, Dordrecht, Netherlands.

Kornberg A (1974) DNA synthesis. 2. Auflage, W.H. Freeman and Company, San Francisco, pp. 29-54.

Kotani Y, Yamaguchi K, Kato F, Furuya A (1978) Inosine accumulation by mutant of *Brevibacterium ammoniagenes* strain improvement and culture conditions. Agric. Biol. Chem. 42: 399-405.

Kraulis P J (1991) MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. 24: 946-950.

Kulkarni AD, van Buren CT, Rudolph FB (1998) Ribonucleotide preparations and uses thereof. US patent 5,712,256.

Kuninaka A (1986) Nucleic acids, nucleotides, and related compounds. *In*: Rehm HJ, Reed G (eds.) Biotechnol. vol. 4: Microbial products II. Weinheim: VCH Verlagsgesellschaft, pp. 71-117.

Kuninaka A (1996) Nucleotides and related compounds. *In*: Roehr M (ed.), Products of primary metabolism, vol. 6. VCH, Weinheim, Germany, pp. 561–612.

Kurnasov OV, Polanuyer BM, Ananta S, Sloutsky R, Tam A, Gerdes SY, Osterman AL (2002) Ribosylnicotinamide kinase domain of NadR protein: identification and implications in NAD biosynthesis J. Bacteriol. 184: 6906-6917.

Lanzillo JJ (1990). Preparation of digoxigenin-labeled probes by the polymerase chain reaction. Biotech. 8: 620-622.

Latch J, Margolin W (1997) Generation of buds, swellings, and branches instead of filaments after blocking the cell cycle of *Rhizobium meliloti*. J. Bacteriol. 179: 2373-2381.

Lengeler JW, Drews G, Schlegel G (1999) Biology of the prokaryotes. Georg Thieme Verlag, Stuttgart, Germany.

Levin, PA, Shim JJ, Grossman AD (1998) Effect of *minCD* on FtsZ ring formation and polar septation in *Bacillus subtilis*. J. Bacteriol. 180: 6048-6051.

Lillehoj EP, Ford GM (2000) Industrial biotechnology, overview. *In*: Lederberg J (ed.) Encyclopedia of microbiology, 2nd edn. Academic Press, New York, pp 722-737.

Lioyd DH, Lamport AI, Noble WC, Howell SA (1999) Fluoroquinolone resistance in *Staphylococcus intermedius*. Veterinary Dermatology 10: 299-251.

Lukacin R, Gröning I, Pieper U, Matern U (2000) Site-directed mutagenesis of the active site serine290 in flavanone 3b-hydroxylase from *Petunia hybrida*. Eur. J. Biochem. 267: 853-860.

Luo C, Hansen J, Auling G (1997) Temperature-sensitive mutants of *Corynebacterium ammoniagenes* ATCC 6872 with a defective large subunit of the manganese-containing ribonucleotide reductase. Arch. Microbiol. 167: 317-324.

Lutkenhaus J, Addinall, SG (1997) Bacterial cell division and the Z ring. Annu. Rev. Biochem. 66: 93-116.

MacAdams HH, Shapiro L (2003) A bacterial cell-cycle regulatory network operating in time and space. Science 301: 1874-1877.

Maessen JG, van der Vusse GJ, Vork M, Kootstra G (1988) Nucleotides, nucleosides, and oxypurines in human kidneys measured by use of reversed-phase high-performance liquid chromatography. Clin. Chem. 34: 1087-1090.

Maga JA (1994) *Umami* flavour in meat. *In*: Shahidi F (ed.) Flavor of meat and meat products. Chapman & Hall, Glasgow, pp 98-115.

Malumbres M, Gil JA, Martin JF (1993) Codon preference in corynebacteria. Gene 134: 15-24.

Martin JF, Gil JA (1999) Corynebacteria. *In*: Demain AL, Davies JE (eds.) Manual of industrial microbiology and biotechnology. 2nd edn. ASM Press, Washington, DC. pp 379-391.

Maxwell A (1999) DNA gyrase as a drug target. Biochem. Soc. Trans. 27: 48-53.

McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F, Hou S, Layman D, Leonard S, Nguyen C, Scott K, Holmes A, Grewal N, Mulvaney E, Ryan E, Sun H, Florea L, Miller W, Stoneking T, Nhan M, Waterston R, Wilson RK (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413: 852-856.

McCormick JR, Su EP, Driks A, Losick R (1994) Growth and viability of *Streptomyces coelicolor* mutant for the cell division gene *ftsZ*. Mol. Microbiol. 14: 243-254.

Merritt E A, Bacon DJ (1997) Raster3D: Photorealistic molecular graphics. Methods Enzymol. 277: 505-524.

Mihara Y, Utagawa T, Yamada H, Asano Y (2000) Phosphorylation of nucleosides by a mutated acid phosphatase from *Morganella morganii*. Appl. Environ. Microbiol. 66: 2811-2816.

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press. New York.

Miwa K, Matsui H, Terabe M, Makamori S, Sano K, Momose H (1984) Cryptic plasmids in glutamic acid-producing bacteria. Agric. Biol. Chem. 48: 2901-2903.

Mori H, Iida A, Fujio T, Teshiba S (1997) A novel process of inosine 5′-monophosphate production using overexpressed guanosine / inosine kinase. Appl. Microbiol. Biotechnol. 48: 693-698.

Moriya S, Imai Y, Hassan AK, Ogasawara N (1999) Regulation of initiation of *Bacillus subtilis* chromosome replication. Plasmid 41: 17-29.

Nagodawithana TW (1994) Savory flavors. *In*: Gabelman A (ed.) Bioprocess production of flavor, fragrance, and color ingredients. John Wiley & Sons Inc. New York, pp 135-168.

Nakata K, Inui M, Kos P, Vertes AA, Yukawa H (2003) Vectors for genetic engineering of

corynebacteria. *In*: Saha BC (ed.) ACS Symposium Series 862. American Chemical Society, Washinton, DC. pp175-191.

Nakayama K, Sato Z, Tanaka H, Kinoshita S (1968) Production of nucleic acid-related substances by fermentative process. Part XVII. Production of NAD and nicotinic acid mononucleotide with *Brevibacterium ammoniagenes*. Agric. Biol. Chem. 33: 1198-1204.

Nanninga N (2000) Cell division, prokaryotes. *In*: Lederberg J (ed.) Encyclopedia of microbiology, 2nd edn. Academic Press, New York, pp 704-709.

Nara T, Komuro T, Misaway M, Kinoshita S (1969) Production of nucleic acid-related substances by fermentative processes. Part XXXIX. Growth responses of *Brevibacterium ammoniagenes*. Agric. Biol. Chem. 33: 1030-1036.

Nishio Y, Nakamura Y, Kawarabayasi Y, Usuda Y, Kimura E, Sugimoto S, Matsui K, Yamagishi A, Kikuchi H, Ikeo K, Gojobori T (2003) Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of *Corynebacterium efficiens*. Genome Res. 13: 1572-1579.

Noguchi Y, Shimba N, Kawahara Y, Suzuki E, Sugimoto S (2003) ^{31}P NMR studies of energy metabolism in xanthosine-5′-monophosphate overproducing *Corynebacterium ammoniagenes*. Eur. J. Biochem. 270: 2622-2626.

Oehlmann W, Auling G (1999) Ribonucleotide reductase (RNR) of *Corynebacterium glutamicum* ATCC 13032 – genetic characterization of a second class IV enzyme. Microbiol. 145: 1595-1604.

Oehlmann, W. (1998) Klonierung der Gene der Ribonucleotid Reduktase von *Corynebacterium ammoniagenes* und *Corynebacterium glutamicum*. Ph.D. thesis, Hannover University, Germany.

Oehlmann, W., Griepenburg, U. & Auling, G. (1998) Cloning and sequencing of the *nrdF* gene of *Corynebacterium ammoniagenes* ATCC 6872 encoding the functional metallo-cofactor of the manganese-ribonucleotide reductase (Mn-RNR). Biotechnol. Lett. 20: 483-488.

Ogata K, Kinoshita S, Tsunoda T, Aida K (1976) Microbial production of nucleic acid-related substances. Halsted Press, New York.

Ohnishi J, Mitsuhashi S, Hayashi M, Ando S, Yokoi H, Ochiai K, Ikeda M, (2002) A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Appl. Microbiol. Biotechnol. 58: 217-223.

Oka T, Udagawa K, Kinoshita S (1968) Unbalanced growth death due to depletion of Mn^{2+} in *Brevibacterium ammoniagenes*. J. Bacteriol. 96: 1760-1767.

Paesi-Toresan SO, Maris AF, Brendel M, Henriques JAP (1998) The *Saccharomyces cerevisiaegene PSO5/RAD16* is involved in the regulation of DNA damage-inducible genes *RNR2* and *RNR3*. Curr Genet 34: 124-127

Pichoff S, Vollrath B, Bouche JP (1997) MinCD-independent inhibition of cell division by a protein that fuses MalE to the topological specificity factor MinE. J. Bacteriol. 179: 4616-4619.

Pogliano J, Pogliano K, Weiss DS, Losick R, Bechwith AJ (1997) Inactivation of FtsI inhibits constriction of the FtsZ cytokinetic ring and delays the assembly of FtsZ rings at potential division sites. Proc. Natl. Acad. Sci. USA. 94: 559–564.

Pühler A (1993) Genetic engineering of microorganisms. VCH, Weinheim, Germany.

Pühler A, Tauch A (2003) A new era in *Corynebacterium glutamicum* biotechnology. J. Biotechnol. 104: 1-3.

Ramos A, Adham SAI, Gil JA (2003a) Cloning and expression of the inorganic pyrophosphatase gene from the amino acid producer *Brevibacterium lactofermentum* ATCC 13869. FEMS Microbiol. Lett. 225: 85-92

Ramos A, Honrubia MP, Valbuena N, Vaquera J, Mateos LM, Gil JA (2003b) Involvement of DivIVA in the morphology of the rod-shaped actinomycete *Brevibacterium lactofermentum*. Microbiol. 149: 3531-3542.

Reineccius G (1994) Source book of flavors. 2nd edn. Chapman & Hall, New York.

Rothfield LI, Justice SS (1997) Bacterial cell division: The cycle of the ring. Cell 88: 581-584.

Rothfield LI, Justice SS, Garcia-Lara J (1999) Bacterial cell division. Annu. Rev. Genet. 33: 423-448.

Rothfield LI, Zhao CR (1996) How do bacteria decide where to divide? Cell 84: 183-186.

Rowland SL, Katis VL, Partridge SR, Wake RG (1997) DivIB, FtsZ and cell division in *Bacillus subtilis*. Mol. Microbiol. 23: 295-302.

Sackett JM, Kelly AJ, Brun YV (1998) Ordered expression of *ftsQA* and *ftsZ* during the *Caulobacter crescentus* cell cycle. Mol. Microbiol. 28: 421-434.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Santamaria R, Gil JA, Mesas JM, Martin JF (1984) Characterization of an endogenous plasmid and development of cloning vectors and transformation system in *Brevibacterium lactofermentum*. J. Gen. Microbiol. 130: 2237-2246.

Shi H, Ho CT (1994) The flavour of poultry meat. *In*: Shahidi F (ed.) Flavor of meat and meat products. Chapman & Hall, Glasgow, pp 52-70.

Shiio I, Nakamori S (1970) Microbial production of L-threonine. Part II. Production by α-amino-β-hydroxyvaleric acid resistant mutants of glutamate producing bacteria. Agric. Biol. Chem. 34: 448-456

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Gen. Microbiol. 135: 675-682.

Spratt BG (1975) Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc Natl Acad Sci USA 72: 2999-3003.

Stubbe J (1990) Ribonucleotide reductases: Amazing and confusing. J. Biol. Chem. 265: 5329-5332.

Stubbe J, Ge J, Yee CS (2001) The evolution of ribonucleotide reduction revisited. Trends Biochem. Sci. 26: 93-99.

Stubbe J, van der Donk WA (1995) Ribonucleotide reductases: Radical enzymes with suicidal tendencies. Chem. Biol. 2: 783-801.

Stubbe J, van der Donk WA (1998) Protein radicals in catalysis. Chem. Rev. 98: 705-762.

Sun Q, Margolin W (1998) FtsZ dynamics during the division cycle of live *Escherichia coli* cells. J. Bacteriol. 180: 2050-2056.

Switzer R, Zalkin H, Saxild HH (2002) Purine, pyrimidine, and pyridine nucleotide metabolism. *In*: Sonenshein AL, Hoch JA, Losick R (eds.) *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press. Washington, D.C., pp 255-269.

Tao H, Bausch C, Richmond C, Blattner FR, Conway T (1999) Functional genomics: Ex-

pression analysis of *Escherichia coli* growing on minimal and rich media. J. Bacteriol. 181: 6425-6440

Tauch A, Pühler A, Kalinowski J, Thierbach G (2003) Plasmids in *Corynebacterium glutamicum* and their molecular classification by comparative genomics. J. Biotechnol. 104: 27-40.

Teshiba S, Furuya A (1982) Mechanism of 5′-inosinic acid accumulation by permeability mutants of *Brevibacterium ammoniagenes*. I. Genetical improvement of 5′-IMP productivity of a permeability mutant of *B. ammoniagenes*. Agric. Biol. Chem. 46: 2257-2263.

Teshiba S, Furuya A (1983) Mechanism of 5′-inosinic acid accumulation by permeability mutants of *Brevibacterium ammoniagenes*. II. Sensitivities of a series of mutants to various drugs. Agric. Biol. Chem. 47: 1035-1041.

Teshiba S, Furuya A (1984) Mechanism of 5′-inosinic acid accumulation by permeability mutants of *Brevibacterium ammoniagenes*. IV: Extraction mechanism of 5′-IMP. Agric. Biol. Chem. 48: 1311-1317.

Teshiba S, Furuya A (1989) Production of nucleotides and nucleosides by fermentation. Amsterdam: Gordon and Breach Science Publishers, OPA BV.

Torrents E, Aloy P, Gibert I, Rodriguez-Trelles F (2002) Ribonucleotide reductases: divergent evolution of an ancient enzyme. J. Mol. Evol. 55: 138-152.

Torrents E, Roca I, Gibert I (2003) *Corynebacterium ammoniagenes* class Ib ribonucleotide reductase: transcriptional regulation of an atypical genomic organization in the *nrd* cluster. Microbiol. 149: 1011-1020.

Towbin HG, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354.

Trusca D, Scott S, Thompson C, Bramhill D (1998) Bacterial SOS checkpoint protein SulA inhibits polymerization of purified FtsZ cell division protein. J. Bacteriol. 180: 3946-3953.

Uppsten M, Färnegårdh M, Jordan A, Eliasson R, Eklund H, Uhlin U (2003) Structure of the large subunit of class Ib ribonucleotide reductase from *Salmonella typhimurium* and its complexes with allosteric effectors. J. Mol. Biol. 330: 87-97.

van Loon APGM, Hohmann HP, Bretzel W, Hümbelin M, Pfister M. (1996) Development of a fermentation process for the manufacture of riboflavin. Chimica 50: 410-412.

Vanderhaegen B, Neven H, Coghe S, Verstrepen KJ, Derdelinckx G, Verachtert H (2003) Bioflavoring and beer refermentation. Appl. Microbiol. Biotechnol. 62: 140-150.

Vicente M, Gomez MJ, Ayala JA (1998) Regulation of transcription of cell division genes in the *Escherichia coli dcw* cluster. Cell. Mol. Life Sci. 54: 317-324.

Viereck HO (1975) Fermentation von *Brevibacterium ammoniagenes* und Untersuchung der extrazellulären Enzyme der NAD-Biosynthese. Ph.D. thesis, Hannover University, Germany.

Voet D, Voet JG, Pratt CW (1999) Fundamentals of biochemistry. John Wiley & Sons, Inc. New York.

Wehmeier L, Schäfer A, Burkovski A, Krämer R, Mechold U, Malke H, Pühler A, Kalinowski J (1998) The role of the *Corynebacterium glutamicum rel* gene in (p)ppGpp metabolism. Microbiol. 144: 1853-1862.

Weiss DS, Chen JC, Ghigo J, Boyd D, Beckwith AJ (1999) Localization of FtsI (PBP3) to the septal ring requires its membrane anchor, the Z Ring, FtsA, FtsQ, and FtsL. J. Bacteriol. 181: 508-520.

White D (2000) The physiology and biochemistry of prokaryotes. 2nd edn. Oxford University Press, New York.

Willi K, Sandmeier H, Kulik EM, Meyer J (1997) Transduction of antibiotic resistance markers among *Actinobacillus actinomycetemcomitans* strains by temperate bacteriophages AaΦ23. Cell Mol. Life Sci. 53: 904–910.

Willing A, Follmann H, Auling G (1988) Nucleotide and thioredoxin specificity of the manganese ribonucleotide reductase from *Brevibacterium ammoniagenes*. Eur. J. Biochem. 175: 167-173.

Woldringh CL, Odijk T (1999) Structure of DNA within the bacterial cell: physics and physiology. *In*: Charlebois RL (ed.) Organization of the prokaryotic genome. American Society for Microbiology, Washington, DC, pp 171-187.

Wynants J, van Belle H (1985) Single-run high-performance liquid chromatography of nucleotides, nucleosides, and major purine bases and its application to different tissue extracts. Anal. Biochem. 144: 258-266.

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 287-294.

Zellner G, Geveke M, Macario ECD, Diekmann H (1991) Population dynamics of biofilm development during start-up of butyrate-degrading fluidized-bed reactor. Appl. Microbiol. Biotechnol. 36: 404-409.

Zhou P, Bogan JA, Welch K, Pickett SR, Wang HJ, Zaritsky A, Helmstetter CE (1997) Gene transcription and chromosome replication in *Escherichia coli*. J. Bacteriol. 179: 163-169.

ACKNOWLEDGMENTS

CURRICULUM VITAE

Personal details

Name	**Hesham** Mohsen Ali **Elhariry**
Sex	Male
Date of birth	27.05.1969
Place of birth	Cairo, Egypt
Nationality	Egyptian
Marital status	Married, with three children

Education

1975-1980	Primary School, Cairo, Egypt
1980-1983	Elementary School, Cairo, Egypt
1983-1986	Secondary School, Cairo Egypt
1986-1990	Bachelor of Food Science, Department of Food Science, Ain Shams University, Cairo Egypt
1992-1996	Master of Science, Department of Food Science, Faculty of Agriculture, Ain Shams University, Cairo, Egypt
12. 2000- 05.2004	Ph.D student, Institute of Microbiology, Hannover University, Germany.

Working

1991-1996	Administrator, Department of Food Science, Faculty of Agriculture, Ain Shams University, Cairo, Egypt
1996-	Lecturer assistant, Department of Food Science, Faculty of Agriculture, Ain Shams University, Cairo, Egypt