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Microbial competition and mixed substrate utilisation in the laboratory: towards a better understanding of microbial behaviour in the environment

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presented by
Hans Peter Füchslin
Dipl. Umweltnaturwissenschafter ETH Zürich
born March 29, 1970, in Brugg, Switzerland

on the recommendation of
Prof. Dr. A.J.B. Zehnder, examiner
Prof. Dr. W. Verstraete co-examiner
PD Dr. T. Egli, co-examiner

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Für meine lieben Eltern

Ruth

und

Marcel (1931-2000)

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Abbreviations and symbols

μ :	specific growth rate [h^{-1}]
μ_{max} :	maximum specific growth rate [h^{-1}]
μ_{maxGFP}:	maximum specific growth rate [h^{-1}] of GFP strain with 2,4-D
μ_{maxWT}:	maximum specific growth rate [h^{-1}] of wild type with 2,4-D
2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DNP	2,4-dinitrophenol
D:	dilution rate [h^{-1}]
D_{crit}:	critical dilution rate (at which cells are wash-out)
DOC	dissolved organic carbon
DW	dry weight
F	flow rate [$l \cdot h^{-1}$]
HPLC	High-pressure liquid chromatography
K_s	Monod half saturation constant [$\mu g \cdot l^{-1}$]
K_{sGFP}:	Monod half saturation constant [$\mu g \cdot l^{-1}$] of GFP strain for 2,4-D
K_{sWT}:	Monod half saturation constant [$\mu g \cdot l^{-1}$] of wild type for 2,4-D
lac(con) mutant	mutant of *Escherichia coli* expressing the *lac* operon constitutively
OD	optical density
s:	steady-state substrate concentration in continuous culture [$\mu g \cdot l^{-1}$]
s(0):	concentration at time 0
s(t):	concentration at time t
s_{min}:	predicted substrate concentration at D= 0 h^{-1}
s_o	substrate concentration in the medium feed
t:	time
V:	working volume of the bioreactor [l]
X	biomass
x	biomass concentration
$Y_{X/S}$:	growth yield [gram dry biomass formed · gram substrate consumed^{-1}

Summary

In the natural environment microorganisms are in constant mutual competition for the uptake and consumption of nutrients. Particularly heteroptrophic microbes are exposed to a wide range of compounds that can serve as potential carbon and energy sources. However, environmental concentrations of microbiologically utilisable substrates are usually very low, and in virtually all ecosystems growth of heterotrophs is slow and for most of the time severely limited by the availability of carbon/energy sources. To survive and compete successfully for nutrients in such an environment the kinetic properties and performance of microbial cells are of primary importance. Recent observations suggest that under carbon-energy-limited growth conditions most microbial cells will probably utilise mixtures of carbon/energy sources for growth rather than single compounds. However, little experimental data is still available on the kinetics and competition of microbial strains for mixtures of substrates during slow growth. To better understand and predict the behaviour of microbial strains under complex growth conditions the kinetics of growth and competition for single and defined mixtures of substrates in pure and mixed cultures of three selected bacterial strains with different growth properties was investigated in this thesis under controlled conditions. The strains included the enterobacterium *Escherichia coli*, as a typical r-strategist, and the two environmentally abundant and successful pollutant-degrading gram-negative k-strategists *Chelatobacter heintzii* and *Ralstonia eutropha*.

To study the competition of *E. coli* with *C. heintzii* for glucose in carbon-limited chemostat culture at low dilution rates the kinetic parameters of the two strains, i.e., the maximum specific growth rate (μ_{max}, the Monod saturation constant (K_s) and the minimum concentration required for growth (s_{min}), were first determined. The specific affinity ($\mu_{max}\ K_s^{-1}$) of both strains was very similar (0.0094 $h^{-1}\cdot\mu g^{-1}\cdot l$ for *E. coli* and 0.011 $h^{-1}\cdot\mu g^{-1}\cdot l$ for *C. heintzii*, respectively) but the two strains differed with respect to their s_{min} required for growth. Whereas *E. coli* exhibited an enhanced s_{min} of ca. 22 $\mu g\cdot l^{-1}$ at 30°C, s_{min} was undetectably low for growth of *C. heintzii* with glucose. This advantage of *C. heintzii* to compete for glucose was

confirmed in glucose-limited chemostat cultures at D = 0.05 and 0.075 h^{-1} where *E. coli* was out-competed consistently. Using the experimentally determined growth parameters the ability of *E. coli* to compete with *C. heintzii* was modelled in dependence of temperature and for fluctuating glucose concentrations. The results indicate that *E. coli* was able to compete successfully only at temperatures above 29°C. The model also predicted a successful competition of *E. coli* during fluctuating environmental glucose concentrations. The results suggest the existence of a distinct s_{min} as the main competitive disadvantage of *E. coli* under the growth conditions tested.

As a second system the growth of the pesticide (2,4-D) degrading bacterium *Ralstonia eutropha* and a mutant strain harbouring a as a reporter system was investigated. When the wild type *R. eutropha* was cultivated with fructose only, or with mixtures of fructose and 2,4-D in carbon-limited chemostat culture at D = 0.075 h^{-1}. During growth with fructose only the expression of all *tfd* genes was low. Exposure of the culture to 0.1mM 2,4-D resulted in the instantaneous increase in mRNA levels of these genes and in the subsequent simultaneous utilisation of 2,4-D and fructose. Mixed 2,4-D/fructose utilisation occurred independent of the mixture composition and growth yields were additive. During mixed substrate growth the steady-state residual concentrations of the two carbon sources were proportional to their fraction in the feed medium, hence, simultaneous utilisation of fructose and 2,4-D allowed degradation of 2,4-D to lower concentrations at a set dilution rate.

Green-fluorescent protein (GFP) harbouring constructs are frequently used as marker and reporter systems to assess the fate and activity of microbial strains with the ability to degrade xenobiotic chemicals. To evaluate the potential of this tool for tracking pesticide-degrading strains in the environment a GFP reporter system lined to genes coding for the degradation of 2,4-D was integrated into the chromosome of *R. eutropha*. In batch culture with 2,4-D as the only source of carbon and energy μ_{max} of the wild type and GFP clone were identical. However, compared to the wild type, the 2,4-D steady state concentration in a 2,4-D-limited chemostat culture of the GFP clone was higher at all dilution rates tested, demonstrating a reduced affinity for the pollutant. The kinetics of both wild type

and the GFP clone can be described with a Monod model extended by s_{min}. The reduced competitiveness of the GFP clone was confirmed in 2.4-D-limited continuous culture at D = 0.075 h^{-1} where the mutant was reproducibly out-competed by the wild type strain. This is the first time that the integration of a GFP reporter system has been shown to lead to a negative impact on the growth kinetics of the construct.

As a third example, the utilisation of mixtures of glucose and lactose by *E. coli* was investigated in carbon-limited chemostat at D = 0.075 h^{-1}, where a short-term and a long-term response was documented. The short-term response of a culture growing with glucose as the primary carbon source was characterised by a threshold concentration of the utilisation of lactose. Wen the concentration of this sugar remained below 2 $mg \cdot l^{-1}$ no utilisation was observed. Challenging the culture with higher lactose concentrations resulted in the induction of enzymes of the *lac* operon (followed by monitoring the activity of β-galactosidase) and subsequent simultaneous utilisation of glucose and lactose. Long-term exposure to lactose concentrations below the threshold did result in the selection of *lac* constitutive mutants and a segregation of the culture into a lactose- and a glucose-utilising sub-population. The *lac* constitutive mutant population displaced the wild type strain when lactose contributed to a large part of the total sugar mixture. The better competitiveness of the *lac* constitutive mutant population was based on its simultaneous utilisation of glucose and lactose, resulting in the ability to grow at the set dilution rate at lower residual sugar concentrations.

Zusammenfassung

Mikroorganismen in der Umwelt stehen in dauernder gegenseitiger Konkurrenz bei der Aufnahme von Substraten. Heterotrophe Mikroorganismen sind verschiedensten Substraten ausgesetzt, welche als Kohlenstoff- und Energiequelle dienen können. Die Umweltkonzent-rationen dieser Substrate sind sehr tief und in scheinbar allen Ökosystemen ist das Wachstum von heterotrophen Organismen ausserordentlich langsam und für die meiste Zeit limitiert durch die Verfügbarkeit von Kohlenstoff- und Energiequellen. Unter solchen kargen Lebensbedin-gungen sind die wachstumskinetischen Eigenschaften für die Weiterexistenz von entscheidender Bedeutung. Experimentelle Erfahrungen der letzten Jahre belegen, dass unter kohlenstofflimitierten Bedingungen die meisten Mikroorganismen die Mischsubstrat-verwertung der Einzelsubstratverwertung vorziehen. Es sind aber nur wenige experimentelle Daten verfügbar über die Kinetik und Kompetition von auf Einzel- und Substratgemischen langsam wachsenden Mikroorganismen. Um das mikrobiologische Verhalten unter komplexen Umweltbedingungen besser zu verstehen, wurde in dieser Arbeit die Wachstums- und Kompetitionskinetik von reinen und definierten Mischkulturen auf Einzel- und Mischsubstraten wachsend untersucht. In den Experimenten wurden 3 Bakterien mit verschiedenen wachstumskinetischen Eigenschaften verwendet. Das Enterobakterium *Escherichia coli*, als typischer r-Stratege und zwei in der Umwelt häufig vorkommende, Schadstoffabbauer und K-Strategen *Chelatobacter heintzii* und *Ralstonia Eutropha*.

Es wurde die Kompetition von *E. coli* mit *C. heintzii* für Glukose in kohlenstofflimitierten kontinuierlichen Kulturen studiert. Zuerst wurden die kinetischen Parameter der 2 Stämme experimentell bestimmt (die maximale spezifische Wachstumsrate (μ_{max}), die Affinitätskonstante (K_s) und die minimal erforderliche Wachstumskonzentration (s_{min})). Die spezifische Affinität ($\mu_{max} \cdot K_s^{-1}$) der beiden Stämme war sehr ähnlich (0.0094 $h^{-1} \cdot \mu g^{-1} \cdot l$ für *E. coli* und 0.011 $h^{-1} \cdot \mu g^{-1} \cdot l$ für *C. heintzii*). Sie unterschieden sich aber in der minimal erforderlichen Wachstumskonzentration (s_{min}). Während für *E. coli* ein s_{min} von 22.3 $\mu g \cdot l^{-1}$ bei 30°C bestimmt werden konnte, war im Falle von *C. heintzii* s_{min} so tief für

Glukose, dass er nicht nachgewiesen werden konnte. Entsprechend gewann *C. heintzii* gegenüber *E. coli* deutlich in den Kompetitionsexperimenten in glukoselimitierten kontinuierlichen Kulturen bei einer Verdünnungsrate von 0.05 h^{-1} und 0.075 h^{-1}. In Computersimulation unter Anwendung der experimentell bestimmten kinetischen Parameter wurde die Möglichkeit der Koexistenz von *E. coli* unter schwankenden Umweltglukosekonzentrationen und Konkurrenz von *C. heintzii* geprüft. Dabei wurde die unterschiedliche Temperaturabhängigkeit der kinetischen Parameter berücksichtigt. Bei einer Temperatur ab 29°C und höher war *E. coli* im kompetitiven Vorteil, währenddessen bei tiefen Temperaturen *C. heintzii* gewinnen würde. Der wichtigste kompetitive Nachteil von *E. coli* beruht unter den getesteten Wachstumsbedingungen auf dem Vorhandensein eines signifikanten $s_{min.}$

Als ein zweites System wurde das Wachstum von dem 2,4-D Pestizid abbauenden Bakterium *R. eutropha* und dem aus ihm abgeleiteten Konstrukt mit integriertem Reportersystem untersucht. Während Wachstum mit Fruktose alleine in kohlentstoffimitierter kontinuierlicher Kultur war die Expression aller *tfd* Gene tief. Wurde die Kultur 0.1 mM 2,4-D ausgesetzt, resultierte dies in einem schnellen Anstieg der mRNA-Level der 2,4-D Abbaugene und in der nachfolgenden simultanen Verwertung von 2,4-D und Fruktose. Die simultane Verwertung war unabhängig von der Mischsubstratzusammensetzung (2,4-D/ Fruktose) und die Ausnützungskoeffizienten waren additiv. Die Restkonzentration der zwei Kohlenstoffquellen richteten sich proportional zu ihrem Anteil im zugeführten Medium. Es konnte somit bei einer simultanen Verwertung von 2,4-D/Fruktose und gegebener Verdünnungsrate die Restkonzen-trationen gesenkt werden.

Um die Aktivität von Bakterien in der Umwelt zu messen, werden vermehrt Reportersysteme wie das green fluorescent protein (GFP) eingesetzt. In so genannten GFP – Konstrukten werden die Expression der Abbaugene mit der Expression von GFP gekoppelt. Es stellte sich die Frage in wie weit der Einbau von fremden Genen die Abbaukinetik eines Bakteriums beeinträchtigt. Es wurde deshalb die Wachstumskinetik von *Ralstonia eutropha* mit 2,4-D zwischen dem Wildtyp und dem daraus entwickelten GFP-Konstrukt verglichen. In

Wachstumsexperimenten mit Batchkulturen wurden statistisch nicht unterscheidbare spezifische maximale Wachstumsraten für GFP-Konstrukt wie für Wildtyp gemessen, währenddessen in kontinuierlichen Kulturen bei allen Verdünnungsraten der GFP-Konstrukt durchgehend eine höhere 2,4-D Restkonzentration aufwies als der Wildtyp. Die Kinetik des Wildtypus und des GFP Stammes konnte mit der Monodkinetik beschrieben werden, welche mit einem Term für eine minimale Substratkonzentration für das Wachstum (s_{min}) erweitert wurde. In Kompetitionsexperimenten in 2,4-D limitierter kontinuierlicher Kultur konnte bei einer Verdünnungsrate von D=0.075 h^{-1} die kinetische Benachteiligung des GFP- Klones bestätigt werden. Der GFP-Klon wurde von dem Wildtyp klar verdrängt.
Es konnte zum ersten Mal gezeigt werden, dass der Einbau eines GFP-Gens einen negativen Einfluss auf die Wachstumskinetik eines Bakteriums hat.

Als ein drittes Beispiel wurde die Mischsubstratkinetik von *E. coli* bezüglich Gemischen von Glukose/Laktose in kohlenstofflimitierten kontinuierlichen Kulturen bei einer Verdünnungsrate von 0.075 h^{-1} untersucht. Es konnte zwischen einer kurzfristigen und einer langfristigen kinetischen Reaktion unterschieden werden. Wurde eine *E. coli*- Population auf Glukose wachsend kurzfristig Laktose ausgesetzt, so musste eine Schwellenwert von 2 $mg \cdot l^{-1}$ erreicht sein damit das Laktose Operon der ganzen Population induziert wurde (gemäss den β-Galaktodosidasemessungen) und Glukose und Laktose gleichzeitig aufgenommen und metabolisiert wurden. Unter dem Schwellenwert war kein Abbau von Laktose zu verzeichnen. Bei einer langfristigen Exposition unterhalb des Schwellenwertes von 2 $mg \cdot l^{-1}$ wurde die Laktose erst metabolisiert, wenn sich Mutanten selektioniert hatten, welche konstitutiv das *lac* Operon exprimierten. Die Population trennte sich auf in spezialisierte Subpopulation für die Laktose- und Glukosekonsumation. Die *lac* konstitutiven Mutanten verdrängte den Wildtyp bei einem erhöhten Anteil von Laktose im Medium. Der kompetitive Vorteil des *lac* konstitutiven Mutanten lag in der gleichzeitigen Verwertung von Glukose und Laktose, welches ein Wachstum bei tieferen Zuckerrestkonzentrationen bei der gegebenen Verdünnungsrate ermöglichte.

1. General introduction

1.1 Growth conditions in the environment

In any ecosystem the carbon contained in living cells will sooner or later end up as dead organic matter, detritus. Heterotrophic microorganisms, by mineralising detritus derived from primary and secondary biomass, serve as a crucial link in global carbon cycling. Detritus and its degradation products are traditionally divided into a particulate and a dissolved organic carbon fraction (POC, DOC). In aquatic systems the two fractions are defined arbitrarily as the organic material either being retained by or passing a filter of 0.2 to 0.45 μm (Williams, 1986). Not only POC but also a large fraction of the DOC is present in a polymeric form. Some of the major components that have been identified include cellulose, lignin, chitin, hemicellulose, lignocelluloses, proteins, inulin, nucleic acids, polyphenols, waxes, tannin, and melanin, but there are many others more, as well as many (70-90%) that have not yet been identified (Williams, 1986; Nedwell & Gray, 1987; Beauchamp et al., 1989; Münster & Chrost, 1990). Because microorganism are only able to transport monomers and oligomers in the range of a few hundred daltons across the cell envelope, only a small fraction of the total DOC (0.5-5%), usually referred to as assimilable organic carbon, can be readily utilised by microorganism (Münster, 1993). Easily degradable carbohydrates are mostly found at very low concentrations (1-200$\mu g \cdot l^{-1}$) and are produced and partly excreted by algae and plants during the light phase (Münster, 1993). They can be directly utilised but seem to be a relevant source of carbon only in highly productive aquatic systems (Jackson, 1987; Münster & Chrost, 1990; Williams, 1990). Hence, the major part of the potential carbon substrates in DOC first has to be hydrolysed outside of the cell and is therefore not immediately available for microbial growth.

In addition to the natural global biogeochemical carbon cycle, the flux of synthetically produced organic materials in industrialised countries has increased over the last two centuries to up to approximately 40 g of carbon per m^2 per annum. This figure is equivalent to approximately 15% of the net primary biomass production in these regions (Egli, 1992). 100 000 synthetic chemicals are

known to be produced today in different quantities. Annually 1000 new compounds come on the market and are released into the environment (European Environment Agency, 1998).

Hence, the scenario outlined above indicates that in the environment microbial cells are exposed to a wide range of different potential carbon/energy sources, including chemical pollutants that are extremely low in concentration, typically in the nanogramm per litre or, at the most, a few hundred $\mu g \cdot l^{-1}$ range (Münster, 1993). Many heterotrophic and also autotrophic microbes are competing for this scarce resources and, as a result, grow only at low rates or are in a starvation diet (Morita, 1997). One can argue that microorganisms themselves are responsible for the oligotrophic conditions found in most ecosystems because they have evolved high affinity uptake systems for most of the natural carbon/energy sources. Only in the intestine of mammals, on decomposing cadavers, or on plant surfaces eutrophic conditions can be found (Morita, 1997). But here growth is frequently limited by the availability of other nutrients than carbon or terminal electron acceptors (Egli, 1995).

Under such growth conditions a successful growth strategy for a competitive heterotroph would be to take up as many as possible of the different available carbon/energy substrates at the same time (Lendenmann, 1994; Egli, 1995) in order to satisfy the requirement for maintenance energy and to achieve the highest possible growth rate. Indeed, in the environment the simultaneous utilisation of different carbon/energy substrates is thought to be the rule rather than the exception (Egli, 1995) and this behaviour is referred to as "mixed substrate growth" (Harder & Dijkhuizen, 1976; Egli, 1995).

Not only in ecological niches but also in man-made industrial systems such as activated sludge reactors and industrial bioprocesses mixtures of substrates will be metabolised by the microbial populations. These technical ecosystems are usually optimised for a specific purpose, e.g. high pollutant removal or product formation rates. The rational design of industrial bioprocesses is therefore also intricately linked to a better understanding of mixed substrate growth. Hence, the phenomenon of mixed substrate growth is of ecological and industrial importance.

1.1.1 Biodegradability and bioavailability

The different carbon compounds are classified into readily biodegradable, potentially biodegradable and persistent compounds (OECD, 1981; Wagner, 1988). Readily biodegradable compounds can be utilised quickly by metabolically active microbial communities whereas in the case of potentially biodegradable compounds utilisation is observed only after a certain period of adaptation. Persistent compounds cannot be degraded by microorganism, even after an extended adaptation period (Morita, 1997). Furthermore, one has to distinguish between bioavailability and biodegradability. Certain compounds may be biodegradable but cannot be metabolised by microbial cells because they are complexed with humic acids sorbed to other organic and inorganic matter (Alexander, 1994; Morita, 1997).

1.1.2 Laboratory growth conditions and the way to more "ecological" experiments

For defined and reproducible laboratory studies microbiologist grow pure cultures in synthetic media, which are usually designed such that all essential nutrients are in the form of single components, e.g. glucose is supplied as a sole carbon and energy source, ammonia is the only source of nitrogen, and so on. Normally the strains are grown in batch culture or in continuous cultures at relatively high dilution rates ($> 0.2\ h^{-1}$) in order to get analysable substrate concentration above the detection limit. Such cultivation conditions contrast sharply with the growth environment microorganism encounter in ecosystems. Mixed populations of different strains are growing mostly very slowly on an enormous spectrum of potential substrates at low concentrations and are in competition with each other for the scarcely available carbon sources. In this study it was tried to design laboratory experiments in a more "ecological" way by increasing step by step their complexity in a defined way starting out from growth of pure cultures with pure substrates during slow growth, to growth of pure cultures with mixed substrates and then to competition of defined mixed cultures for single and mixed carbon sources. With this approach it is hoped to obtain information on the principles of growth kinetics and physiology that are governing microbial growth and biodegradation of chemicals in the environment.

1.2 Single substrate kinetics

Microbial growth kinetics describes the relationship between the specific growth rate (μ) and the concentration of a growth-limiting substrate (s). It has been observed that μ increases with s in a "saturation-type"– like dependence until bacteria attain their maximum specific growth rate. Until today the relatively simple empirical relationship proposed by Monod (Monod, 1942) is the most widely used equation (1) to describe the kinetics of microbial growth.

$$\mu = \mu_{max} * \frac{s}{s + K_s} \qquad (1)$$

Where μ_{max} is the maximum specific growth rate of the bacterium or the culture/population and K_s is the saturation constant. Various other models or modifications of the Monod model have been proposed, which in specific cases can describe some experimental data equally well or even better (see appendix, Tables 1 and 2). The success of the Monod model is partly due to its simplicity, the similarity to the well-established enzyme kinetics (Michaelis-Menten kinetics), biologically meaningful interpretation of the model constants μ_{max} and K_s, and their experimental accessibility.

In this study, mainly the Monod model expanded by s_{min} was used in addition to the classical Monod model. The extended form of the Monod equation (2) predicts a finite threshold substrate concentration at zero growth rate. The existence of s_{min} can be justified on the basis of the maintenance energy concept as originally proposed by Pirt (Pirt, 1965) and has been verified recently for growth of *E. coli* with glucose (Kovarova et al., 1996a). It is especially important at low growth rates and substrate concentrations that are normally found in the environment.

$$\mu = \mu_{max} * \frac{(s - s_{min})}{(s - s_{min}) + K_s} \qquad (2)$$

Unfortunately, kinetic constants are not always "constant". There are several reasons for this variability (Grady et al., 1996). The most important reasons are different growth conditions (temperature, oxygen concentration, pH, etc.) and culture history (Kovarova & Egli, 1998). Also, microbial cells can adjust their kinetic parameters as a result of physiological adaptation. For example, in a short-term response they can switch between two or more substrate transport systems of different affinity (Harder & Dijkhuizen, 1982; Tros et al., 1996). Furthermore, when cultivated for extended periods in the same environment they can adapt by mutation and selection to the particular environmental conditions given (Hartl & Dykhuizen, 1979; Wick et al., 2001). So far, the simple kinetic model proposed by Monod to describe the complex metabolic process of microbial growth has been studied under well defined laboratory conditions, i. e., single substrates as carbon and energy source (Monod, 1942). It remains to be tested whether or not these models can describe microbial growth in nature where microorganisms are exposed to and are utilising probably not only one but a wide range of different carbon sources.

1.3 Microbial growth with mixtures of substrates: An overview

The general phenomenona and definitions

For understanding growth and degradation processes in ecological and industrial systems the mechanism have to be studied and understood that govern growth with mixtures of substrates.

When considering microbial growth with mixtures of substrates two types have to be distinguished. First, mixtures can consist of homologous substrates for example carbon sources, that serve the same nutritional function (Harder & Dijkhuizen, 1976). When each component of such a mixture supports single substrate growth they are referred to as substitutable nutrients (León & Tumpson, 1975). Second, substrates that serve different nutritional requirements for the cell, for example, as a carbon or as a nitrogen source, are called heterologous substrates (Harder & Dijkhuizen, 1982). In this case both nutrients are essential for growth.

The substrate excess conditions in batch culture are quite different from the carbon-limited growth conditions in the continuous culture. Different patterns and strategies of mixed substrate utilisation have been reported for these two methods of cultivations and these will be discussed below.

1.3.1 Effects observed in batch culture

Under typical batch growth conditions, where carbon sources are supplied at concentrations of grams per litre, diauxic or sequential utilisation of mixtures of carbon substrates is a widespread phenomenon (Monod, 1942). Many organisms first use the substrate that supports the highest maximum specific growth rate while the utilisation of other substrates remains repressed. However, also the simultaneously utilisation of carbon substrates is frequently observed (Pineault et al., 1977; Hegewald & Knorre, 1978; Brooke & Attwood, 1983; Krauel et al., 1984; de Boer et al., 1990; Kastner & Roberts, 1990; Wanner & Egli, 1990; Degnan & Mcfarlane, 1993). In this case preferred substrates are usually utilised at higher rates than other substrates. It was demonstrated in some cases that microorganism can increase their maximum specific growth rate in batch culture when they grow on a mixtures of carbon sources compared to growth with one of the substrates alone (Wood & Kelly, 1977; Cognan, 1987; Loubiére & Lindley, 1991; Loubiére et al., 1992). Another important phenomenon reported for growth with carbons source mixtures is the observation that the threshold concentration below which microorganism cannot grow appears to be lower than during growth with single carbon substrates (Law & Button, 1977; Van der Kooij et al., 1982; Kovarova & Egli, 1998). For example *Pseudomonas* was reported to grow with a mixture of 45 different carbon compounds, each added to tap water at a concentration of 1 $\mu g \cdot l^{-1}$ of carbon, whereas none of these compounds supported growth on their own at this concentration (Van der Kooij et al., 1982).

1.3.2 Effects observed in continuous culture

Simultaneous utilisation „Mixed substrate growth“

Mixtures of carbon substrates provoking diauxic growth in batch culture are usually utilised simultaneously in carbon-limited continuous culture at low dilution rates (Harder & Dijkhuizen, 1976). Many catabolic enzymes that are repressed during batch cultivation are expressed under slow growth conditions in

the chemostat even if the appropriate carbon sources are absent (Egli et al., 1980; Gottschal et al., 1981; Sepers, 1984; Schultz et al., 1988; Blum et al., 1990; Wick et al., 2001). It seems a reasonable reasonable strategy of carbon/energy-limited cells to widen their catabolic potential to be able to scavenge new carbon sources, even if they are present only at low concentrations. At enhanced dilution rates, repression the utilisation of the secondary substrate by the primary substrate can take place such that they are not utilised anymore (Mateles et al., 1967; Eggeling & Sahm, 1981; Harder & Dijkhuizen, 1982; Egli et al., 1983; Egli et al., 1986). An important observation was made for mixed substrate growth in carbon-limited chemostat cultures for various organisms and carbon substrate mixtures, namely that the steady-state concentrations of a particular substrate became reduced during mixed-substrate growth conditions (Egli et al., 1983; Babel et al., 1993; Weusthuis et al., 1993). In addition the critical dilution at which the cells are washed out could be increased compared to the single substrate utilisation (Dijkhuizen & Harder, 1979; Egli et al., 1982; Egli et al., 1986; Kysliková & Volfová, 1990; Loubiére et al., 1992; Lendenmann, 1994). The most complete kinetic analysis of mixed substrate growth was published by Lendenmann and co-workers for growth of *E. coli* with mixtures of sugars. The authors demonstrated that the steady-state concentrations of individual sugars were proportional to their contribution to the total substrate concentration in the feed (Egli et al., 1993; Lendenmann et al., 1996). The data strongly suggest that even when these sugars are present at low concentrations they can support a growth rate much higher than the one that could be achieved during growth with a single sugar meanwhile this strategy/kinetic principle has been confirmed for a number of combinations of different substrates and organisms (Kovarova & Egli, 1998).
Selection of mutants during prolonged growth in chemostat culture is a well-established phenomenon. During extended growth with single, limiting substrates often mutants arise with increased levels of an enzyme involved in the metabolism of the growth-limiting nutrient (Novick & Szilard, 1950; Mateles et al., 1967; Savva, 1982; Ferenci, 1996; Wick, 2002).

The two strategies

Hence in summary, for a population cultivated with mixture of substrates two principally different ways exist to adapt. Firstly, every cell may use

simultaneously all the available substrates and cells optimise their mixed substrate utilisation behaviour (Bally et al., 1994; Lendenmann & Egli, 1997). Secondly mutants may be selected in the population with an enhanced affinity to one of the substrates, e. g. by enhancing the expression of uptake proteins. This will lead to a segregation of the originally homogenous population into two specialised subpopulations (Kaprelyants et al., 1993).

1.3.3 Kinetic models for mixed substrate utilisation

Based on available experimental data we are far from being able to formulate general models for the kinetics during growth of microbial cells with mixtures of substrates under different environmental conditions. This is reflected in the wide range of different kinetic models, which are only partially able to explain the observed mixed substrate phenomena. The models can be divided into unstructured empirical models and physiologically structured models (Narang, 1998). Empirical models usually assume that the specific growth rate for each substrate follows Monod kinetics, or some modification of it and the interaction between the substrates is mutually inhibitory (see appendix, Table 3). Most of these models contain no upper limit for μ_{max}, which is a serious drawback. This implies that an increasing number of simultaneously uses will result in an increasingly higher $\mu_{max,}$ until it becomes unrealistic (Kovarova & Egli, 1998). Since unstructured models either lack physiological variables or, if present, have no effect on the specific growth rate, they provide no insight into the physiological basis of growth. Several physiologically structured models were proposed and developed into increasingly complex kinetics (Nielsen & Villadsen, 1992; Bailey, 1998). But always it remains a difficult task to experimentally determine realistic values of the different physiological parameters used in these kinetic models. In specific cases it was possible to apply the models proposed to the chosen conditions but it was not possible to generalise their application. Furthermore, a specific kind of physiological models based on a cybernetic approach has recently been put forward, which assumes that the description of regulatory mechanisms can be replaced by optimality principles that microbes follow (Turner et al., 1988; Baloo & Ramkrishna, 1991). However, also these models still await experimental verification and validation.

1.4 Competition

Microorganism is not only subjected to a range of different physiochemical influences but they are also affected by the activities of other microorganisms present in the same environment.

The probably most important biotic interaction is the competition for common substrates, such as energy and carbon sources (Gottschal, 1993). The competition determines not only the composition of the microbial population but also the concentration of growth-limiting substrates in the environment and is therefore of crucial importance for microbial processes in ecosystems. The simplest case is a "pure and simple" competition amongst microbial strains. A competition has been defined as pure and simple (Frederickson & Stephanopoulos, 1981) if the availability of only one nutrient affects the growth rate ('simple') and if this is the only interaction ('pure') between competitors. Although it is questionable whether this 'pure' and 'simple' type of competition is very common in nature it represents by far the most extensively experimentally studied interaction amongst microorganisms in the laboratory. Although the growth behaviour of microbes is coupled and numerous concepts for general principles have been put forward (Gottschal, 1993), the most successful of which is the concept of the opportunist and the gleaner (Veldkamp & Jannasch, 1972; Grover, 1990). With respect to the competition for a common substrate it will be shortly presented here:

Opportunists have a high maximum growth rate (μ_{max}) but low substrate affinity (high K_s). Due to the high maximum growth rate carbon sources can be utilised in an opportunistic way if they come rapidly and abundantly available by a sudden change of environmental conditions either due to a flash of nutrients or a rapid elimination of competitors. In contrast, gleaners operate in oligotrophic ecosystems with scarce organic resources. Their main catabolic characteristics are increased effectiveness of nutrient assimilation and so-called efficient, or economical, metabolism. Therefore, they have a high competitiveness at low substrate concentrations, which has its expression in a high substrate affinity (low K_s) and a very low or undetectable low s_{min}. However, concerning the maximum specific growth rate (μ_{max}) they are inferior to opportunists.

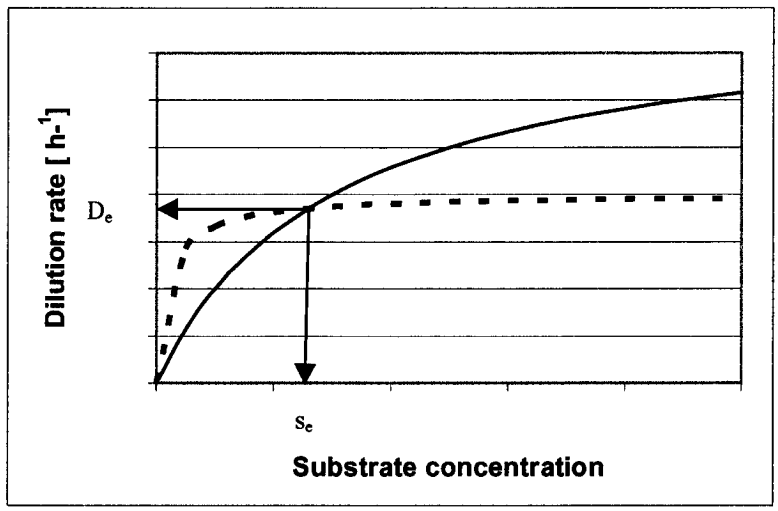

Fig. 1.1 μ-s relationship of two organism A(—) and B(·····). Microorganism A with low substrate affinity (high $K_{s(A)}$) and high μ_{max} is called an opportunist, a copiotrophic strain or a r-strategist, whereas microorganism B with high substrate affinity (= low $K_{s(B)}$) and low μ_{max} is called a gleaner, oligotrophic strain or a k-strategist. B will outcompete A at $s<s_e$, whereas A will outcompete B at $s>s_e$. Coexistence is theoretically possible at D_e, where the growth rates of the two strains are equal.

This situation is depicted in Fig. 1.1 for a model gleaner and opportunist system. In a continuous culture the opportunist will win a single substrate-limited competition at high dilution rates ($D>D_e$) whereas at low dilution rates ($D<D_e$) it will be outcompeted by the gleaner. At the dilution rate $D=D_e$, coexistence of the two strains is theoretically possible. However, the random variations of physical parameters such as flow rate and culture volume make such a coexistence rather unlikely (Stephanopoulos et al., 1979).

Nevertheless, coexistence in pure and simple competition for one substrate is possible when either the substrate concentration in the feed (Hsu, 1980; Smith, 1981; Hale & Somolinos, 1983) or the dilution rate is varying (Grenney et al., 1973; Stephanopoulos et al., 1979). In such a system, both the residual concentration of the limiting substrate and the biomass concentration of the two strains are fluctuating and if the cycles are periodic the system is exhibiting cyclic oscillations (Frederickson & Stephanopoulos, 1981). Other bacterial interactions can also lead to stable mixed cultures and to deviations from the pure competition behaviour. An example is the excretion of a metabolic by-product, that supports the growth of an inferior competitor for the common substrate (Gottschal et al., 1979). It is also possible that inhibitory or even toxic substance are excreted that

have a negative effect on an otherwise superior competitor, a phenomenon that was referred to as competitive inhibition by a product (Pirt, 1975).
When mixtures of substrates are fed the system can support the coexistence at maximum the same number of different strains as the number of different substrates fed, unless additional interactions are involved (Frederickson & Stephanopoulos, 1981; Gottschal, 1993). Generalists able to utilise simultaneously different substrates should have a competitive advantage because they are able to lower the residual concentrations of individual substrates during mixed substrate growth (Egli et al., 1993). This again should lead to a reduction of the strains able to coexist in such a system.

1.5 Goal of this thesis

Substrate utilisation, growth processes and microbial competition are fundamental aspects of microbiology. To date kinetic models derived from simple idealised single substrate laboratory systems are used to describe and predict these processes in the environment. However, conditions in the environment are far more complex and it is questionable whether or how the presently available models can be applied to ecosystems and man-made complex systems such as wastewater treatment plants. One important difference is the fact that in the environment microorganisms are exposed to a wide range of highly diluted potential carbon/energy sources and not to single substrates at high concentrations.
Therefore, as a first step towards a better understanding of the complex growth processes in the environment we set out to study the growth of defined bacterial populations with defined mixtures of carbon sources under controlled experimental conditions.
As model systems the behaviour of *E. coli* was studied during growth with mixtures of glucose and lactose in continuous culture giving particular attention to the mixed substrate kinetics, the composition and selection dynamics of the wild type and *lac*(con) mutant population. This classic example of mixed substrate utilisation was compared with the mixed substrate utilisation of the established herbicide degrader *Ralstonia eutropha* on a mixture of fructose and the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). The kinetics for the 2,4-D-degradation was compared between the *Ralstonia eutropha* wild type and a

genetic engineered construct containing a green-fluorescent protein as a reporter system. Furthermore, the simple and pure competition for glucose between an enterebacterium *Eschericha coli* ML30, and the environmentally successful bacterium *Chelatobacter heintzii* in continuous culture at different dilution rates was studied.

2. Growth of *E. coli* K12 with mixtures of glucose and lactose in continuous culture: Mixed substrate utilisation and population segregation

Hans Peter Füchslin and Thomas Egli

Abstract

Typical for the growth conditions encountered by microorganisms in most environmental and technical compartments is the presence of mixtures of utilisable carbon sources. Therefore, a key for understanding microbial processes in biological and engineered systems is the knowledge of the principles of mixed substrate utilisation and growth kinetics.

As a model system the kinetic behaviour of a wild type population of *Escherichia coli* cultivated with mixtures of lactose and glucose was elucidated in a carbon-limited continuous culture at a fixed dilution rate of $D=0.075\ h^{-1}$. This experimental system was chosen because preliminary observations indicated that the utilisation of glucose and lactose could result from either simultaneous utilisation of the two sugars by the whole population, or the segregation of the population into a glucose-utilising and a lactose-utilising subpopulation. In this work the dependence of the two processes on the glucose/lactose mixtures in the feed medium and the time of exposure to the two sugars was investigated. The short-term response to mixtures of glucose and lactose was investigated by either pulsing a culture growing in a glucose-limited chemostat with a high concentration of lactose or by shifting a glucose-limited culture to a medium containing different mixtures of glucose and lactose.

When a culture of the wild type cultivated with glucose was shifted to a medium containing defined mixtures of glucose plus lactose for less than approximately 20 generations no utilisation of lactose by the wild type was observed when the

concentration of lactose in the medium remained below the threshold of 2 $mg \cdot l^{-1}$ (For the long-term response and selection of lactose constitutive mutants at lactose concentrations < 2 $mg \cdot l^{-1}$, see chapter 2). When the threshold lactose concentration was exceeded by pulsing the glucose-limited culture with excess lactose, by shifting the culture to a medium with excess lactose, or by shifting the culture to a medium with sugar mixtures containing more than 2 $mg \cdot l^{-1}$ lactose the whole population became induced for β-galactosidase and the wild type strain grew simultaneously with glucose and lactose. No accumulation of *lac* constitutive mutant was detected in these short-term experiments. In medium-shift experiments a threshold concentration of 2 $mg \cdot l^{-1}$ for lactose utilisation and a K_s for lactose of 76.8 $mg \cdot l^{-1}$ was determined for the wild type strain of *E. coli* K12.

Long-term cultivation of the wild type with mixtures of glucose and lactose feed concentration (> 2 $mg \cdot l^{-1}$) in a carbon-limited chemostat resulted in the selection of a population consisting exclusively of lactose constitutive mutants and the elimination of the wild type from the culture. The enriched mutant population simultaneously utilised the two sugars and exhibited the typical mixed substrate kinetic pattern previously also observed for the growth with defined mixtures of carbon sources in C-limited continuous culture of other microbial strains and substrate mixtures.

Introduction

Information on the physiological and kinetic behaviour of microorganisms is usually derived from experiments where the culture is grown in defined synthetic media in which all essential nutrients are supplied in the form of single compounds. For example, glucose is supplied as the only carbon source, ammonia as the only source of nitrogen, and so on. This allows controlling the growth in laboratory experiments and to obtain clear – cut reproducible data. However, such cultivation conditions contrast sharply with the growth environment microorganisms encounter in natural and technical systems, where cells are usually exposed to a multiplicity of compounds that can serve the same physiological function (Egli, 1995; Morita, 1997; Egli, 2000). In the case of heterotrophic microorganisms this holds especially for carbon/energy sources. Despite this, understanding of microbial function and behaviour is typically deduced from single substrate growth experiments. For example, the kinetic equations used today to describe microbial growth in nature are still based on the assumption that the extracellular concentration of a single substrate (s) is governing the specific growth rate (μ). The most widely used model is the saturation kinetics (equation 1) originally proposed by Monod (Monod, 1942), where K_s is the saturation constant and μ_{max} is the maximum specific growth rate.

$$\mu = \mu_{max} * \frac{s}{s + K_s} \qquad (1)$$

But for a better understanding of microbial degradation and production processes in complex systems more information on the growth of microbial cultures with mixtures of substrates is therefore required, both at the physiological and the kinetic level.

It is well established that in media containing binary mixtures of carbon substrates in excess the consumption of a particular substrate can be repressed by an alternative, easily degradable carbon source. This phenomenon known as catabolite repression, results in a diauxic growth pattern (Monod, 1942; Magasanik, 1961). In *Enterobacteriaceae* glucose is the substrate usually exhibiting the strongest repression effect on the utilisation of other carbon

sources (Stumm-Zollinger, 1966; Postma et al., 1993), whereas di- and tricarbonic acids repress the utilisation of sugars in pseudomonads (Ampe et al., 1998). Less well known is the fact that many micro-organisms do utilise combinations of carbon substrates simultaneously in batch culture even when they are present in excess, particularly when those substrates support only medium or low maximum specific growth rates (for a compilation of data see (Egli, 1995)), and in carbon-limited chemostat culture at low dilution rates the simultaneous utilisation is the rule rather than the exception (Harder & Dijkhuizen, 1976; Egli, 1995; Lendenmann et al., 1996).

One of the best studied mixed substrate systems with respect to the regulation at molecular level is the utilisation of glucose / lactose mixtures by *Escherichia coli*. The *lac* operon consists of three structural genes β-galactosidase, permease and thiogalactosidase transacetylase and their expression is controlled by several mechanisms (Miller & Reznikoff, 1978). A repressor protein (i) exerts negative control by binding tightly and specifically to the operator DNA segment. This strong binding is alleviated by inducer molecules (e.g., by allolactose, a by-product of lactose metabolism) (Jacob & Monod, 1961; Jacob & Monod, 1961; Monod & Jacob, 1961; Beckwith & Zipser, 1970; Wanner, 1975) or a gratuitous inducers such as Isopropyl-1-thio-β-D-galactosidase (IPTG) (Monod, 1956). Expression of the *lac* operon genes requires not only the release of the inactivated repressor protein from the operator but also the presence of the cAMP·CRP complex (Catabolite Repression Protein) a global regulatory mechanism known as carbon catabolite repression (Perlman & Pastan, 1968; Ullmann & Monod, 1968; Notley-McRobb et al., 1997). Furthermore, a mechanism known as inducer exclusion prevents the entry of lactose into the cell and inhibits in such a way the generation of the inducer allolactose (Nelson et al., 1983). It is believed that inducer exclusion is a more powerful control mechanism than catabolite repression. The latter appears to act as a fine-tuning control on the rate of enzyme synthesis (Silver & Mateles, 1969).

In addition to these well characterised mechanisms there seem to be additional factors controlling *lac* operon expression and one speculates over a third mediator of negative control a compound called catabolite modulator (Ullmann et al., 1976). Although the regulation of the *lac* operon is well elucidated concerning the above mentioned regulating mechanisms there is surprisingly little known

about the co-ordination of these mechanisms resulting in the diauxic or simultaneous utilisation of glucose and lactose and the corresponding mixed substrate kinetics. Dependent on the substrate concentration of glucose and/or lactose different utilisation patterns have been reported. Exposing *E. coli* to lactose/glucose mixtures in the $g \cdot l^{-1}$ range results in a diauxic growth pattern, where glucose must be used up before lactose utilisation starts (Monod, 1942). In contrast in carbon-limited culture simultaneous utilisation of glucose and lactose by constitutive mutants has been observed (Silver & Mateles, 1969). These mutants, who constantly express the enzymes necessary for lactose utilisation, appear quickly during glucose/lactose- or lactose-limited growth in the chemostat (Novick & Horiuchi, 1961; Horiuchi et al., 1962).

We therefore set out to study the behaviour of *E. coli* during growth with mixtures of glucose and lactose in continuous culture giving particular attention to the kinetics, adaptation process, and the composition and dynamics of the *E. coli* population with respect to wild type, and *lac*(con) mutants. It was tested whether the simultaneous utilisation of glucose and lactose is only a question of the generation and selection of *lac*(con) mutants or whether also the wild type is able to overcome the repression and to utilise glucose and lactose simultaneously.

Materials and methods

Organism

Escherichia coli K12 (MG 1655) was used in all experiments and is referred as "the wild type".

Medium

The mineral medium contained, per litre: 275 mg NH_4Cl , 1.25 ml H_3PO_4 (85%) , 75 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot H_2O$, 35 mg KCl, 1.5 mg $FeCl_2$, 60 µg H_3BO_3, 100 µg $MnCl_2 \cdot 4H_2O$, 120 µg $CoCl_2 \cdot 6H_2O$, 70 µg $ZnCl_2$, 25 µg $NiCl_2 \cdot 6H_2O$, 15 µg $CuCl_2 \cdot 2H_2O$, 25 µg $Na_2MoO_4 \cdot 2H_2O$, 5.2 mg $EDTA \cdot Na_4(H_2O)_4$.
To one litre of mineral medium 0.25 ml of vitamin stock solution was added (which contained per litre: pyridoxin·HCl, 100 mg; 50 mg of each thiamine·HCl, riboflavin, nicotinic acid, D-Ca-pantothenic acid, p-aminobenzoic acid, lipoic acid, nicotinamide, vitamin B_{12}; biotin 20 mg and folic acid 20 mg). Chemicals were purchased either from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). The mineral medium was supplemented with either glucose, or lactose, or mixtures thereof as only sources of carbon and energy. If not indicated otherwise, the total amount of carbon from the sugars was kept constant at 50 mg carbon per litre.

Cultivation

Continuous cultivation was performed in a bioreactor (MBR, Wetzikon, Switzerland) with a working volume of 2.8 litres. The bioreactor was equipped with both pH (7.50± 0.05) and temperature control (30±0.1°C). The impeller speed was controlled at 1000 revolutions per minute and the oxygen concentration was always > 90% air saturation. Bioreactors were regularly checked for wall growth to avoid artefacts as reported by Pirt (Pirt, 1975).

Lactose analysis

For the analysis of residual lactose concentrations in continuous cultures of *E. coli* the biomass was immediately separated from the culture liquid by filtration as described earlier (Senn et al., 1994). After changing the feed mixture the culture was always allowed to grow for five volume changes before analysis was started (i.e., it may not have reached yet steady-state with respect to all parameters measured!).

Analysis included desalting of samples by electrodialysis and subsequent sugar determination with HPLC separation, post-column reaction with alkaline copper(II)bisphenanthroline and amperometric detection. The method for sugar analysis has previously been published in detail (Senn et al., 1994; Lendenmann, 1994). Unfortunately, for growth experiments with lactose/glucose mixtures it was not possible to determine the residual glucose concentration because lactose eluted too closely to glucose and the peak for glucose was usually hidden below the broad lactose peak caused by the high steady-state lactose concentrations in the range of 1-2 $mg \cdot l^{-1}$. The quantification of lactose, however, was not affected by the presence of glucose because glucose concentrations were always below 20 $\mu g \cdot l^{-1}$ according to model calculations and experimental experience.

Calculation

The dilution rate (D) which under steady state-conditions corresponds to the specific growth rate (μ) in chemostat is defined as

$$D = \frac{F}{V} \tag{2}$$

D: dilution rate $[h^{-1}]$
F: flow rate $[l \cdot h^{-1}]$
V: volume of the reactor [l]

Theoretical wash-out (3) and wash-in curves (4) for concentrations were calculated using the following equations:

$$y(t) = y_0 \cdot e^{-D.t} \quad (3)$$

$$y(t) = y_0 \cdot (1-e^{-D.t}) \quad (4)$$

y_0: concentration at time 0 or in the medium reservoir
y(t): concentration at time t
t: time elapsed [h]

Test for lactose constitutive mutants

Samples taken from the chemostat culture were diluted (factor $2 \cdot 10^5$) in autoclaved phosphate buffered saline (PBS; 14.4 $g \cdot l^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 3.2 $g \cdot l^{-1}$ $NH_2PO_4 \cdot 2H_2O$, 5.9 $g \cdot l^{-1}$ NaCl, pH 7.5) and 100 µl were plated on complex medium agar (containing per litre 4 g of tryptic soy agar (Biolife, Milano, Italy) plus 15 g technical agar Biolife, Milano, Italy)) and plates were incubated overnight at 37°C. Resulting colonies were covered with a drop of toluene, which was allowed to rest for 15 minutes on the colonies. After 15 minutes the cells were lysed and the toluene had evaporated. Then a drop of ONPG (o-nitrophenyl-β-D-galactopyranoside (4 $\mu g \cdot l^{-1}$)) was added onto colonies and colonies of *lac*(con) mutants turned yellow within a few minutes (Dykhuizen & Maxine, 1980).
The percentage of *lac*(con) mutants was calculated using equation 5.

$$\%\ \text{of } lac(\text{con})\ \text{mutants} = 100\% \cdot \frac{\text{number of yellow colonies}}{\text{total number of colonies}} \quad (5)$$

Activity of β-galactosidase

Activity of β-galactosidase was measured according to Miller (Miller, 1977).

Results and discussion

2.1. Effect of increasing proportions of lactose in the feed on lactose utilisation and population composition

E. coli K12 was cultivated in carbon-limited continuous culture at a constant dilution rate of $0.075h^{-1}$ first with glucose only and subsequently with mixtures of glucose and lactose where the proportion of lactose in the mixture was increased stepwise. The effect on the appearance and selection of lactose-constitutive mutants and on residual lactose concentration in the culture was studied (Fig. 2.1).

During growth with glucose the residual glucose concentration was lower than the detection limit of 10 $\mu g \cdot l^{-1}$ and only a low basal level of β-galactosidase of 2.1 Miller units was detected. When this culture was shifted to mixture of 99% of glucose plus 1% of lactose ($s_{0(Lac)}$ = 1.25 $mg \cdot l^{-1}$, equivalent to 0.5 $mg\text{-}C \cdot l^{-1}$). The cells were apparently unable to utilise the lactose presented within five volume changes (equivalent to 7.2 generations). The resulting residual concentration of lactose in the culture corresponded well (± 4%) with the concentration of this disaccharide in the feed (Fig. 2.1c). No *lac*(con) mutants were detected in the population (429 colonies tested). When raising the proportion of lactose to 3% ($s_{0(Lac)}$ = 3.75 $mg \cdot l^{-1}$) the culture utilised approximately 60% of the lactose fed and *lac*(con) mutants appeared in the population (Fig. 2.1c). They contributed to 1.2 ± 0.3 % of the total population (based on total colony count). This figure is slightly lower than the theoretical mutant cell number of 1.8% estimated via the growth yield when assuming that all the lactose utilised was consumed by *lac*(con) mutants only.

The total activity of β-galactosidase of the culture corresponded well with the fraction of *lac*(con) mutants in the population (Fig.2.1a, b). This result suggests that the measured total β-galactosidase activity originated primarily from constitutive mutants and not from wild type cells that became induced, a conclusion that is further supported by the observation that significant utilisation of lactose only started after the appearance of constitutive mutants. Growth with 25% lactose or more resulted in a population that consisted of finally 100% of *lac*(con) mutants. The activity of β-galactosidase of the culture remained constant

at a level of 5844 ± 85 Miller units, which is much higher than the activity of approximately 1000 Miller units of a fully induced wild type culture growing on lactose (Miller, 1977). This result indicates that the selected mutants achieved their increased affinity for lactose by over-expressing the *lac* operon, including the permease, roughly six-fold. It has been reported earlier that in *E. coli* a common way for increasing lactose affinity is to acquire multiple copies of the *lac* operon (Horiuchi et al., 1963; Tisty et al., 1984; Andersson et al., 1998). The fact that the average β-galactosidase activity of constitutive mutants remained at the same level (6304 ± 746 Miller units) during growth with all mixtures indicates that *lac*(con) mutants always exhibited the same expression level and did not evolved further.

Interestingly, the residual concentration of lactose in the culture dropped significantly with increasing proportions of lactose in the feed from 3 to 25% (Fig. 2.1c). At a constant dilution rate the residual lactose concentration should stay constant and independent of the feed concentration according to (Senn et al., 1994; Lendenmann & Egli, 1997).

Hence, this result is in contrast to what one would expect to find for a culture that was in steady-state and consisted of a *lac*(con) mutant population consuming exclusively lactose, and of wild type cells consuming glucose only, both growing at a constant rate of 0.075 h^{-1} set by the dilution rate.

Furthermore, the total dominance of the *lac*(con) mutant during cultivation with 25% or more lactose cannot be explained based on a simple segregation of the culture into a lactose- and a glucose-consuming subpopulation. If this was the case the fraction of wild type cells in the population should decrease linearly with decreasing proportions of glucose. Rather, one has to conclude that the *lac*(con) mutant population competed successfully for glucose with the wild type, and, due to the resulting kinetic advantage from mixed substrate growth (Egli, 1995; Lendenmann et al., 1996), out-competed the wild type. The linearly increasing residual (steady-state) level of lactose during growth with 25 to 100% lactose in the feed parallels the kinetics observed recently during the simultaneous growth of *E. coli* ML30 at a constant dilution rate in the chemostat with combinations of up to six different sugars (Lendenmann et al., 1996) and strongly supports the simultaneous utilisation of glucose and lactose by the selected *lac*(con) mutant.

Three interesting preliminary conclusions can be drawn from this experiment:

I) For a wild type culture of *E. coli* K12 growing at a low dilution rate in continuous culture with glucose a threshold concentration seems to exist that has to be reached until lactose utilisation and the selection of *lac*(con) mutants starts.

II) During growth with glucose/lactose mixtures containing 25% lactose or more the observed lactose kinetics corresponds to the established pure culture mixed sugar kinetics.

III) The observed residual lactose concentrations and population composition during growth with mixtures containing between 3 and 10% of lactose were neither according to those expected for a segregated culture consisting of wild type plus *lac*(con) mutant, each growing with its own sugar, nor did they fit with the mixed substrate kinetic model as found for growth with lactose proportions in the feed >25%. This suggests that under these conditions the population dynamics were slow and the culture had not yet reached its steady-state.

These facts require confirmation and we have therefore investigated these aspects in more detail in a series of experiments.

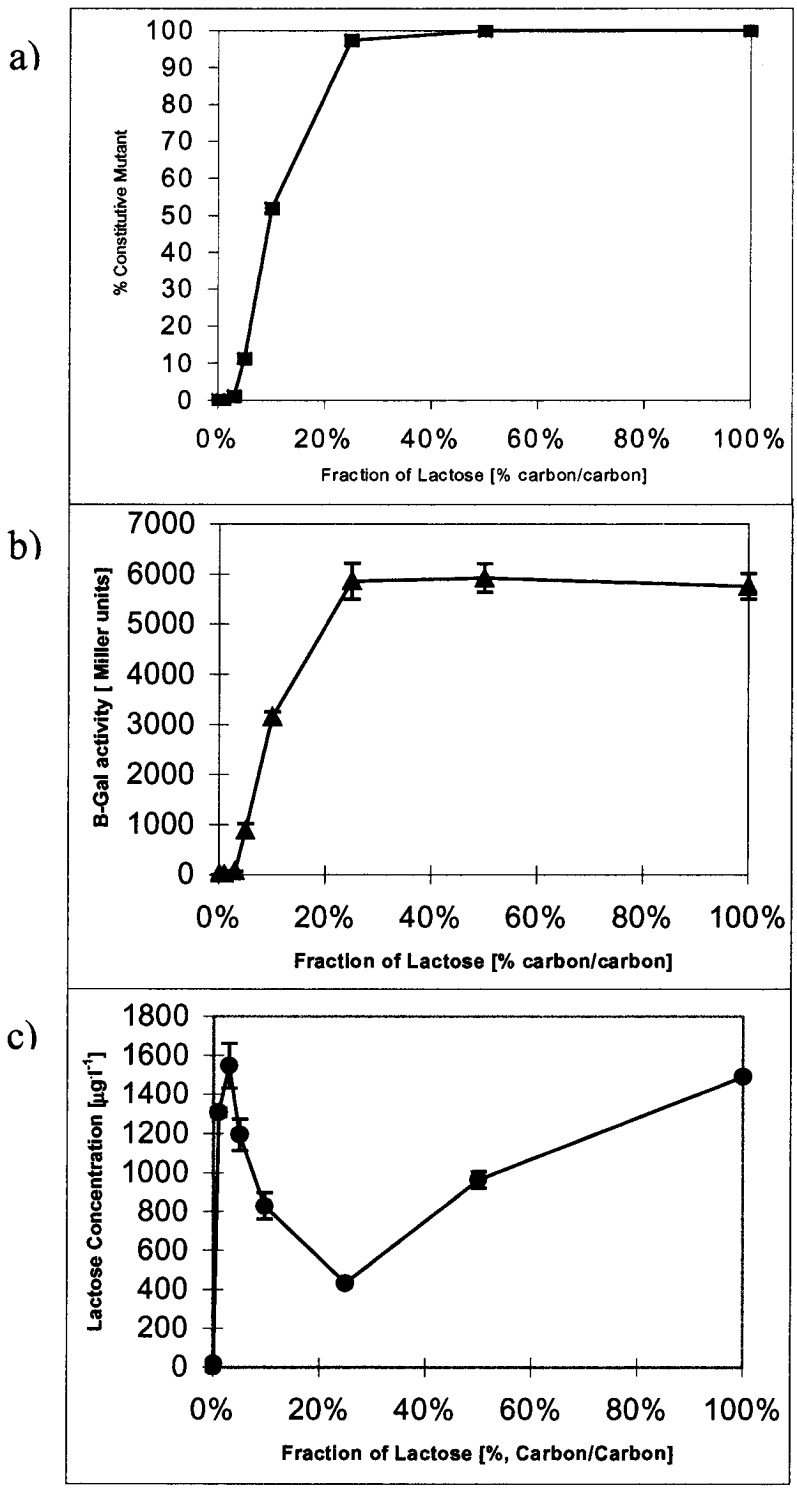

Fig. 2.1 Growth of *E. coli* K12 in carbon-limited continuous culture at a dilution rate of 0.075 h^{-1} with glucose, lactose or mixtures of the two sugars. The culture was cultivated first with glucose only and subsequently with mixtures of glucose and lactose and the proportion of lactose in the mixture was increased stepwise. Throughout the experiment the total concentration of carbon in the feed medium remained constant at 50 $mg \cdot l^{-1}$. The fraction of *lac*(con) mutants in the population (Fig. 2.1a), the total β-galactosidase activity (Fig. 2.1b) and the residual lactose concentration (Fig. 2.1c) was recorded.

Percentage lactose in the medium [%] and feed concentration [$mg \cdot l^{-1}$]	Average β-galactosidase per *lac*(con) mutant	% of the inflowing sugar consumed by *lac*(con) mutants		Lactose residual concentration [$\mu g \cdot l^{-1}$]
		Lactose	Glucose	
0 % (0 $mg \cdot l^{-1}$)	n.a.	n.a.	n.a.	0
0.1 % (0.125$mg \cdot l^{-1}$)	n.a.	n.a.	n.a.	20
1% (1.25 $mg \cdot l^{-1}$)	n.a.	n.a.	n.a.	1306
3% (3.75 $mg \cdot l^{-1}$)	6290	100	0.0	1547
5% (6.25 $mg \cdot l^{-1}$)	7782	64.3	35.7	1194
10% (12.5 $mg \cdot l^{-1}$)	6060	18	82	830
25% (31.25 $mg \cdot l^{-1}$)	6020	25.3	74.7	433
50% (62.50 $mg \cdot l^{-1}$)	5920	49.2	50.8	964
100% (125 $mg \cdot l^{-1}$)	5750	100	0	1492
Average	**6304 ± 746**			

Table 2.1 Estimated sugar consumption by *lac*(con) mutants during growth of *E. coli* K12 in carbon-limited continuous culture with mixtures of glucose plus lactose. The average β-galactosidase activity per *lac*(con) mutants cell was calculated (= β-galactosidase activity·100%/(% *lac*(con) mutants)).
(n.a. = not applicable because no *lac*(con) mutants detected in culture)

2.2 Can the wild type simultaneously utilise lactose and glucose, and is there a threshold concentration for lactose utilisation?

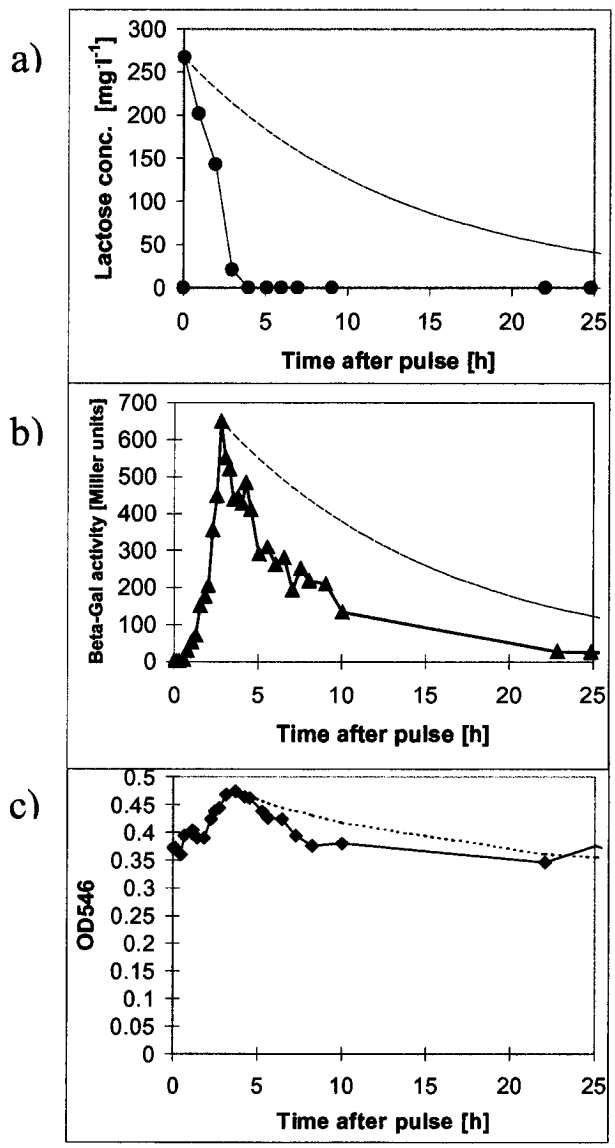

Fig. 2.2 Lactose pulse into a culture of wild type *E. coli* growing in a glucose-limited chemostat (S_0 = 500 mg glucose-C·l^{-1}) at a dilution rate of 0.075 h^{-1}. At time 0 the culture was challenged with a pulse of approximately 300 mg·l^{-1} of lactose without interruption of the glucose feed. Lactose concentration (a), total β-galactosidase activity (b) and biomass concentration given as OD_{546} (c) were monitored as a function of time.

When the wild type culture of *E. coli* K12 was shifted from glucose as the only carbon source to a mixture containing 1.25 $mg \cdot l^{-1}$ lactose (1% of total carbon) the cells were unable to utilise the lactose supplied within ten volume changes (Fig. 2.1). The inability to utilise lactose raises the question of whether or not a threshold lactose concentration must be exceeded before the cells are able to utilise this sugar. Therefore, a culture of wild type cells was exposed to different concentrations of lactose using both pulse and shift experiments. All these experiments were using wild-type cultures that had not been exposed previously to lactose for many generations and the response was followed only over a short time period in order to exclude the generation and selection of a significant number of *lac*(con) mutants. For confirmation, the cultures were always checked for the presence of *lac*(con) mutants and they were always negative (i. e., the population consisted always of > 99.9% of Wild type cells). Throughout the whole experiment 526 colonies were tested and not a single *lac*(con) mutant was detected. The result for one of the pulses performed is shown in. The time course of the concentration of lactose clearly demonstrates that the culture was able to utilise lactose virtually immediately and simultaneously with glucose (Fig. 2.2a). After a short lag of 30 min the total β-galactosidase activity in the culture increased steeply (Fig. 2.2b). Activity of β-galactosidase peaked after some four hours at 650 Miller units, which comes close to the level of a fully induced wild type culture, i.e. approximately 1000 Miller units (Miller, 1977). The specific consumption rate of lactose, $q_{s(lactose)}$ reacted within an hour (0.33 mg substrate·mg dry weight $^{-1}$ ·hours^{-1}), which is twice that of glucose (q_s(glucose)=0.167 mg substrate·mg dry weight $^{-1}$ ·hours^{-1})).

The measured concentrations for lactose indicate that the utilisation of this disaccharide started virtually immediately after the pulse, considerably sooner than enhanced β-galactosidase-activity was detected. This suggests that at high concentrations lactose can be taken up and that the low basic level of lactose-utilising enzymes was sufficient to handle the suddenly appearing high concentration of lactose.

The utilisation of lactose resulted in an increase of the biomass concentration of the culture (Fig. 2.2c). After utilisation of lactose to completion the activity of β-galactosidase decreased significantly faster than washout, indicating a

considerable turnover for this enzyme. By parameter estimation a first order degradation constant for β-galactosidase (0.176 h^{-1}) was determined.

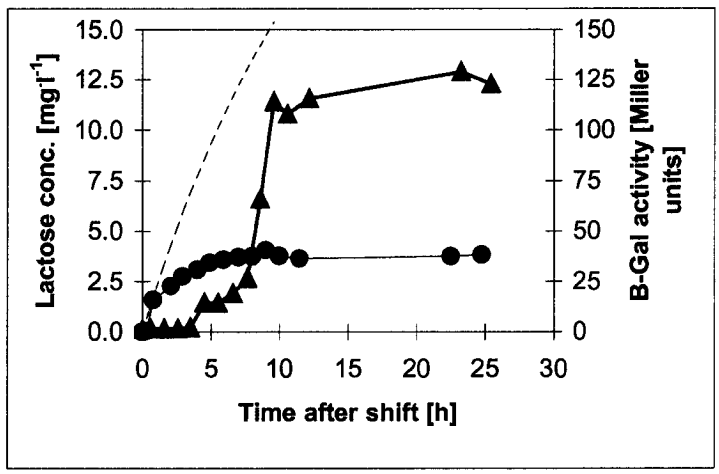

Fig. 2.3 Shift of a culture of wild type *E. coli*, growing in a glucose-limited chemostat, to a medium containing additional lactose (30 mg·l^{-1}).
$S_{0(glucose)}$ was kept constant at 50 mg glucose C·l^{-1}. The dilution rate was 0.075h^{-1} throughout the experiment. Lactose concentration (●) and β-galactosidase activity (▲) was monitored.

An attempt was now made to determine the threshold concentration needed and the time course of the induction of lactose utilisation by exposing a culture growing glucose-limited in shift experiments to continuously increasing concentrations of lactose. A fast (Fig. 2.3) and a slow shift experiment (Fig. 2.4) was performed with different concentrations of lactose added to the glucose feed medium (30 and 3 mg of lactose·l^{-1}, respectively) resulting in different initial rates of increase of the concentration of lactose in the culture.

During the fast shift the activity of β-galactosidase deviated significantly from the basal level of 1.5 Miller units after a lag of 3 hours (Fig. 2.3). During the first two hours after the medium change no lactose was consumed. It increased to a concentration of 1.8 mg·l^{-1} (Fig. 2.3).

Subsequently, the experimentally measured concentrations of lactose in the culture clearly indicate consumption of this sugar. Also here the onset of lactose utilisation occured slightly earlier than the observed appearance of β-galactosidase activity.

Some 8 hours after the shift lactose concentration and β-gal activity established at 3.75 mg·l^{-1} and 120 Miller units, respectively using the mixed substrate kinetics

proposed by Lendenmann (Lendenmann et al., 1996) the K_s for lactose was estimated to be in the range of 77 mg·l^{-1} (equation 6). This is much higher than the K_s of the *lac*(con) mutant (K_s= 6.5 mg·l^{-1}). No *lac*(con) mutants were detected in the population during the whole experiment.

$$K_{s\,lactose} = s_{lactose} * \frac{s_{0lac} + s_{0gluc}}{s_{0lac}} * \frac{(\mu_{max} - D)}{D} \qquad (6)$$

Hence, the results obtained indicate a threshold concentration for induction of lactose metabolism in the range of 1-2 mg lactose·l^{-1}.

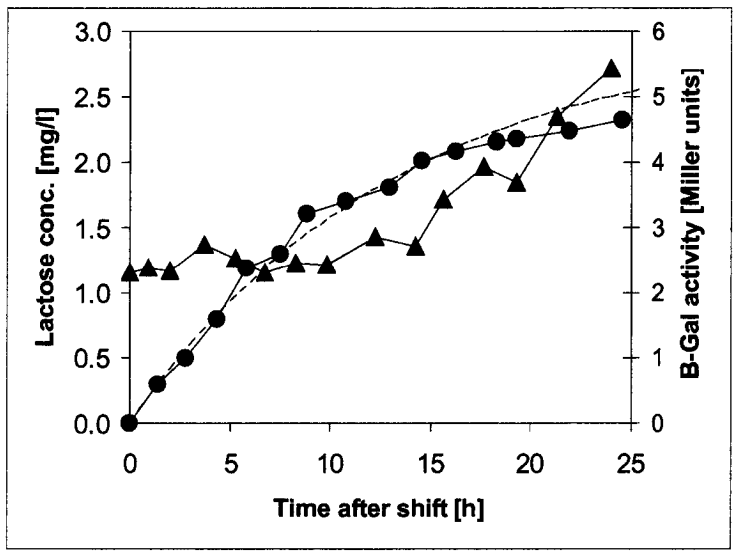

Fig. 2.4 Shift of a culture of wild type *E. coli* growing in a glucose-limited chemostat to a medium containing additional lactose (3 mg·l^{-1}).
s_{0gluc} was kept constant at 50 mg glucose C·l^{-1}. The dilution rate was 0.075h^{-1} throughout the experiment. Lactose concentration (●) and β-galactosidase activity (▲) was monitored.

During the slow shift the concentration of lactose increased steadily and followed the theoretical wash-in curve up to 2 mg·l^{-1}, some 15 hours after initiating the shift (Fig. 2.4). Throughout this time the activity of β-galactosidase remained constant at the low level of 2.4 Miller units (Fig. 2.4). After this time point a clear increase of total activity was observed and, this corresponded nicely with

deviation of lactose concentration from the predicted wash-in curve. No *lac*(con) mutants were detected in the population during the whole experiment.
The two shift experiments demonstrate that wild type cells are able to utilise lactose simultaneously with glucose also when it is supplied in low concentrations. However, for significant short-term induction of the lactose-utilising catabolic pathway (represented here by β-galactosidase) a threshold lactose concentration of at least 1.5-2 $mg \cdot l^{-1}$ has to be exceeded. A similar threshold concentration was reported for *E. coli* for the utilisation of 3-phenylpropionate in the presence of glucose (Kovarova et al., 1997; Kovarova & Egli, 1998). With 3 $mg \cdot l^{-1}$ it was in the same concentration range as the one found here. Both, 3ppa (Kovarova et al., 1996b) and lactose (Monod, 1942) lead to diauxic growth in batch culture when supplied together with glucose.

2.3 Lactose/glucose mixed substrate kinetics exhibited by the *lac*(con) mutant

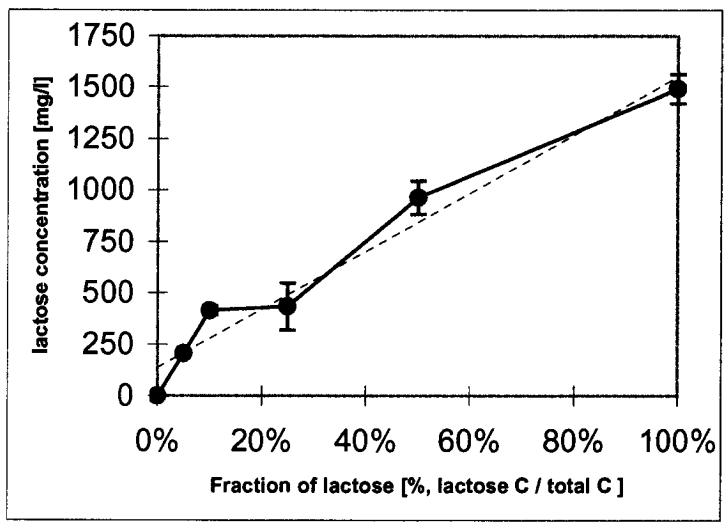

Fig. 2.5 Growth of a *lac*(con) *E. coli* mutant population in carbon-limited continuous culture at a dilution rate of 0.075 h^{-1} with glucose, lactose or mixtures of the two sugars.

A linear correlation between steady-state residual lactose concentration and the proportion of lactose in the feed was observed as soon as the *lac*(con) mutant population had eliminated the wild type population from the chemostat (Fig. 2.1c). This suggests that the steady-state lactose concentrations measured were

essentially a result of "pure culture" kinetics as reported earlier for the growth of *E. coli* with mixtures of glucose and galactose (Egli et al., 1993) or even more complex sugar mixtures (Lendenmann et al., 1996). Hence, for a pure culture of *lac*(con) mutants or even a culture dominated by the *lac*(con) mutant the linear proportional range of steady-state lactose concentrations should be observed also for growth with mixtures containing less than 25% of lactose in the feed. To test this, a culture of *E. coli* was cultivated for an extended time with a mixture of glucose/lactose (50%/50%) until the *lac*(con) mutant dominated the population (>90%). This *lac*(con) mutant population was then shifted to mixture containing different proportions of lactose in the feed and the resulting lactose concentration in the reactor was followed over the first 24 hours after the shift. The residual lactose concentration established after some two hours at a constant value and the results obtained are shown in Fig. 2.5. These lactose concentrations fit well the predicted „pure culture mixed substrate kinetic pattern". Hence, the residual lactose concentration pattern observed in the initial experiment when increasing proportions of lactose from 3% to 25 % were fed did not reflect the steady state situation but were still affected by the unfinished selection process of the competition between the wild type and the *lac*(con) mutant for mixed substrates.

Conclusions

When wild type *E. coli* was cultivated for short periods of time with mixture of glucose/lactose in carbon-limited continuous culture no lactose consumption was observed when concentration of lactose concentration remained below a threshold concentration of approximately 1.5- 2 $mg \cdot l^{-1}$ growing on. This is probably due to inducer exclusion, which prevents the uptake of lactose by inhibition of the lactose permease at low lactose concentrations (Beckwith & Zipser, 1970; Nelson et al., 1983), although a low basal β-galactosidase activity of 2.1 Miller units is always present in the cells indicating that also the other enzymes encoded on the *lac* operon were synthesised at low levels. When exposed to lactose for extended periods of time (> 100 hours, around 11 generations) the population segregated into a wild type and constitutive β-

galactosidase expressing mutant sub-population. The latter was utilising lactose also below 2 $mg \cdot l^{-1}$. The ability to grow with glucose and lactose simultaneously allowed the *lac*(con) mutant to outcompete the wild type strain under mixed substrate growth conditions. This confirms the importance of mixed substrate utilisation for the competition of microbes in the environment.

The growth conditions of glucose/lactose-limited continuous culture here imposed on *E. coli* seem to be rather artificial for *E. coli* strain. Indications for this conclusion come from the observation that *lac*(con) mutants are not selected under natural conditions (Dykhuizen et al., 1987). In contrast to other sugars lactose is virtually found only in milk or dairy products (McGee, 1997). Therefore, only in the intestine *E. coli* should experience lactose as a carbon source. It is hence adapted to pulsed supply similar to our medium-shift and pulse experiments. *E. coli* evolved optimised mixed substrate kinetics for this kind of lactose supply, which is regulated physiologically by induction and repression. The reactions were fast and efficient the bacterial cells expressed β-galactosidase only when it was needed. One can speculate that this regulation this regulation strategy is obviously used for rare sugars such as lactose and arabinose whereas uptake systems for sugars that occur more frequently in the environment are also synthesised in the absence of such sugars (Wick et al., 2001).

3. Dynamics of *lac* constitutive mutant selection during long term exposure of a population of wild-type *E. coli* K12 growing on glucose to low concentration of lactose

Hans Peter Füchslin and Thomas Egli

Abstract

Previous experiments (chapter 1) demonstrated that the short-term induction of the lactose utilising pathway in glucose-growing wild type *E. coli* K12, resulting in a simultaneous utilisation of glucose and lactose, requires a threshold concentration of approximately 2 $mg \cdot l^{-1}$ of lactose to be exceeded. This raises the question whether or not *lac* constitutive mutants are selected when a population of wild-type *E. coli* is exposed to concentrations of lactose below the threshold level needed for immediate induction of the lactose utilising enzyme system. For this, *E. coli* was grown for an extended time period in a carbon-limited continuous culture ($D=0.075\ h^{-1}$) with a medium containing a mixture of 99% glucose and 1% ($1.25\ mg \cdot l^{-1}$) lactose. The residual concentration of lactose, the total β-galactosidase activity in the culture and the percentage of constitutively β-galactosidase expressing mutants (sensitivity of 0.1%) were measured as a function of time. During the first 180 hours (equivalent to 19.5 generations) no detectable lactose consumption was observed. After some 150 hours the first *lac*(con) mutants were detected in the culture and after 180 hours lactose consumption was clearly detectable. As a result, the residual lactose concentration in the culture decreased from $1.25\ mg \cdot l^{-1}$ to $0.4\ mg \cdot l^{-1}$ and the proportion of *lac*(con) mutants in the population increased from undetectable to 7% after some 300 hours of cultivation. Surprisingly, this phase was only transient and as the experiment continued the residual concentration of lactose increased again whereas the part of *lac*(con) mutants decreased. A steady-state

established after some 400 hours of cultivation characterised by a lactose concentration of 900 $\mu g \cdot l^{-1}$ and a fraction of 1% *lac*(con) mutants in the population. The experimental data indicates that the original wild type *E. coli* population was split up by mutation and selection into three different subpopulations consisting of the wild type, a constitutive *lac* operon expressing mutant, and mutants of the wild type with an enhanced affinity for glucose. A kinetic model based on these assumptions fitted the experimental data well. This example demonstrates that an adaptation process in an apparently simple mixed substrate system can not be directly extrapolated from known single substrate properties of the wild type strain. Here, the adaptation processes led to an optimised single substrate uptake by specialised subpopulations but not an optimised mixed-substrate uptake of two sugars by the whole population.

Introduction

Traditional kinetics is based on the assumption that a single compound (for example a carbon source, such as glucose) is controlling the rate of growth of a microbial cell. This concept originally put forward in the forties (Monod, 1942; Hinshelwood, 1946) has been confirmed in a number of laboratory studies (for a summary see (Powell, 1967; Kovarova & Egli, 1998)). But in contrast to such ideal laboratory conditions a microbial cell is exposed in nature to a wide range of different carbon sources (Münster, 1993) and in almost all ecosystems, these carbon sources are present only in very low concentrations and, hence, their availability is extremely restricted (Moriarty & Bell, 1993; Münster, 1993; Morita, 1997). Under such conditions heterotrophic microorganisms do not restrict themselves to the utilisation of a single carbon source but are simultaneously consuming and assimilating different carbon sources, even mixtures of carbon sources that normally provide diauxic growth at high concentrations (Harder & Dijkhuizen, 1976; Matin, 1979; Harder & Dijkhuizen, 1982; Egli, 1995). It has been shown for a number of different organisms that this behaviour, usually referred to a mixed substrate growth, confers some important kinetic advantages to a cell (Kovarova & Egli, 1998). In continuous culture it results in reduced steady-state concentrations of individual substrates compared to the concentration observed under the same conditions with single substrates (Lendenmann & Egli, 1997). Hence, it enables the cell to grow at reasonable rate even when the concentrations of the different individual carbon sources are extremely low and allows achieving the highest possible growth rate under such conditions (Egli, 1995; Lendenmann & Egli, 1997). One can anticipate that this behaviour should result in a competitive advantage compared to an organism growing with a single substrate (Egli, 1995).

In addition to mixed substrate growth a second phenomenon with relevance for the competitive behaviour of microbial strains has been reported in the literature. When cultures of *Cytophaga johnsonae* (Höfle, 1983) or *E. coli* (Kovarova & Egli, 1998; Wick et al., 2001) were exposed to long-term chemostat cultivation under glucose –limited conditions the residual glucose concentration decreased continuously with time and it only became "constant" after some 400-500 generations. A similar behaviour was reported recently also for growth of *E. coli*

with lactose (Tsen et al., 1996). For *E. coli* it was shown recently that mutants with improved glucose affinity were selected in such cultures (Ferenci, 1996; Wick et al., 2001). Wick and co-workers demonstrated that *E. coli* improved its affinity for glucose stepwise during long -term cultivation at dilution rates of 0.1, 0.2 and 0.3 h^{-1} (Wick et al., 2001). This adaptation process was caused by the occurrence of mutants with an average mutation rate of approximately $6 \cdot 10^{-7}$ per cell replication and their subsequent selection. This selection process orchestrated outer membrane changes (lamB) with affinity for glucose with the constitutive expression of the high affinity galactose (mgl) transport system (Ferenci, 1996; Wick et al., 2001). In the study reported by Tsen et al. (1996) lactose mutants occurred after 30 days of cultivation. They exhibited K_s for lactose of 1.4 mM which is half the value of that of the original wild type (Tsen et al., 1996).
The selection of *lac*(con) mutants during chemostat cultivation with lactose alone (Tsen et al., 1996) or with mixtures of glucose and lactose is a well-documented phenomenon (Silver & Mateles, 1969) and three types of *lac*(con) mutants have been reported to appear (Müller-Hill, 1996). However, in the previous chapter evidence was shown that the selection of *lac* (con) mutants might require a lactose concentration exceeding a threshold of approximately 2 $mg \cdot l^{-1}$. When the feed lactose concentration exceeded this threshold *lac*(con) mutants were rapidly selected for and displaced the wild type due to their ability to utilise both lactose and glucose simultaneously. In this chapter it will be investigated whether or not the selection of *lac*(con) mutants also occurs during long-term exposure of a wild type culture growing with glucose to low concentrations of lactose and - if yes - whether or not this also leads to the elimination of the wild type *E. coli* from the culture.

Materials and methods

Organism

Escherichia coli strain K12 (MG 1655) was used in all experiments.

Medium

The mineral medium contained, per litre: 275 mg NH_4Cl , 1.25 ml H_3PO_4 (85%) , 75 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot H_2O$, 35 mg KCl, 1.5 mg $FeCl_2$, 60 µg H_3BO_3, 100 µg $MnCl_2 \cdot 4H_2O$, 120 µg $CoCl_2 \cdot 6H_2O$, 70 µg $ZnCl_2$, 25 µg $NiCl_2 \cdot 6H_2O$, 15 µg $CuCl_2 \cdot 2H_2O$, 25 µg $Na_2MoO_4 \cdot 2H_2O$, 5.2 mg $EDTA \cdot Na_4(H_2O)_4$.
Vitamin stock solution (0.25 ml) was added to 1 litre. The stock solution contained per litre: pyridoxin·HCl, 100 mg; 50 mg of each, thiamine·HCl, riboflavin, nicotinic acid, D-Ca-pantothenic acid, *p*-amino benzoic acid, lipoic acid, nicotinamide, vitamin B_{12}; biotin 20 mg, and folic acid 20 mg. Chemicals were purchased either from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).
The mineral medium was supplemented with a mixture of glucose and lactose as the only sources of carbon and energy. For continuous cultivation the total carbon concentration of the medium was kept at 50 mg carbon per litre. After the initial cultivation in carbon-limited continuous culture at D=0.075 h^{-1} with glucose only (s_0= 125 mg glucose·l^{-1}) the culture was shifted to a medium containing 99% of carbon from glucose (123.75 mg glucose·l^{-1}) plus 1% carbon from lactose (1.25 mg lactose·l^{-1}).

Cultivation

Continuous cultivation at a dilution rate of 0.075 h^{-1} was performed in a bioreactor
(MBR, Wetzikon, Switzerland) with a working volume of 2.8 litre provided with both pH (7.5± 0.05, by automatic addition of 1 M KOH/NaOH) and temperature control (30±0.1°C) was working in chemostatic mode. The impeller speed control was set to 1000 revolutions per minute and the oxygen saturation was always > 90% air saturation. The bioreactors were regularly checked for wall growth to avoid artefacts as reported by Pirt (Pirt, 1975).

Analytical Procedures

Lactose analysis

The biomass was immediately separated from the culture liquid by filtration and the filtrate was used for lactose analysis. Analysis included desalting of samples by electrodialysis and subsequent sugar determination by HPLC separation, post-column reaction with alkaline copper(II)bisphenanthroline and amperometric detection. Further details on the analytical procedures can be taken from the published literature (Senn et al., 1994; Lendenmann & Egli, 1997). Unfortunately it was not possible to quantify the residual glucose concentration. The peak for glucose in the $\mu g \cdot l^{-1}$ range was hidden by the much bigger peak for lactose (typically in the $mg \cdot l^{-1}$ range).

Calculation

The dilution rate, corresponding to the specific growth rate of the culture in a chemostat at steady-state, is defined as

$$D = \frac{F}{V} \qquad (1)$$

D: dilution rate $[h^{-1}]$
F: flow rate $[l \cdot h^{-1}]$
V: volume of the reactor [l]

Test for lactose constitutive mutants

Samples taken from the chemostat culture were diluted ($2 \cdot 10^5$ times) in autoclaved phosphate buffered saline (PBS; 14.4 $g \cdot l^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 3.2 $g \cdot l^{-1}$ $NH_2PO_4 \cdot 2H_2O$, 5.9 $g \cdot l^{-1}$ NaCl, pH 7.5) and 100 µl was plated on complex medium agar (containing per litre 4 g of tryptic soy agar plus 15 g technical agar) plus 15 g technical agar all from (Biolife, Milano Italy)) and plates were incubated overnight at 37°C. Resulting colonies were covered with a drop of toluene, which was allowed to rest for 15 minutes on the colonies. After 15 minutes the cells were lysed and the toluene had evaporated. Then a drop of ONPG (o-nitrophenyl-β-D-galactopyranoside (4mg/ml)) was added onto colonies

and colonies of *lac*(con) mutants turned yellow within a few minutes (Dykhuizen & Maxine, 1980).
The percentage of *lac*(con) mutants was calculated using equation 2.

$$\%\ \text{of } lac(\text{con})\ \text{mutants} = 100\% \cdot \frac{\text{number of yellow colonies}}{\text{total number of colonies}} \qquad (2)$$

Activity of β-galactosidase

Activity of β-galactosidase was measured according to Miller (Miller, 1977).

$$\text{Specific β-gal activity of } lac(\text{con})\ \text{mutants} = \text{total β-galactosidase activity} \cdot \frac{100\%}{\%\ \text{lac(con)mutants}} \qquad (3)$$

Simulation software

The simulation and parameter estimation was carried out using AQUASIM, a program developed at our institute (Reichert, 1998). AQUASIM allows estimating parameters by fitting calculated values (s_{obs}). This feature was used to estimate kinetical parameters, which resulted in the best fit of simulations and measurements. The program estimated the best fit by minimising χ^2 values ($\chi^2=\Sigma((s_{obs}-s_{pred})/\sigma)^2$). For the estimation the standard deviation (σ) was set to 10% if not experimentally determined.

Results

E. coli was pregrown for three successive passages in batch culture on mineral medium containing glucose as the only carbon and energy source. Cells from the exponential phase of the third passage were inoculated into the bioreactor containing a medium with a mixture of 123.75 $mg \cdot l^{-1}$ of glucose plus 1.25 $mg \cdot l^{-1}$ lactose (i.e., 99% of glucose plus 1% of lactose based on carbon). After the culture had reached 60% of its final OD the system was switched to continuous mode with a dilution rate of 0.075 h^{-1}. The lactose concentration in the culture, total β-galactosidase activity and the percentage of constitutive mutants in the population was followed over time (Fig. 3.1).

In the beginning for some 180 hours (19.5 generations) lactose was not utilised by *E. coli,* as indicated by the residual concentration of lactose in the culture, which was equivalent to that in the feed. This was also indicated by the fact that the total β-galactosidase activity remained on a low basal level of 2.5 Miller units and the number of *lac*(con) mutants remained undetectable low i.e. < 0.1%. After 180 hours of cultivation lactose utilisation became detectable and this was linked to an increasing part of the inflowing lactose being consumed, a sharp increase of the percentage of *lac*(con) mutants and a steep increase of the total β-galactosidase activity (Fig. 3.1). After 300 hours, 7% of the population consisted of *lac*(con) mutants. Based on the fact that lactose contributed only to 1% of the inflowing carbon one can conclude that the lac (con) mutants consumed not only lactose but a considerable part of the glucose fed.

However, as the cultivation continued the fraction of constitutive mutants in the population and the total β-galactosidase activity did not stabilise but decreased again to a significantly lower level. Surprisingly, this reduction in the proportion of *lac*(con) mutants in the population was paralleled by an increase in the residual concentration of lactose in the culture. After approximately 500 hours the culture reached a steady-state with respect to all of the parameters measured.

The β-galactosidase activity and the percentage of *lac* (con) mutants seem to correlate closely, suggesting that total β-galactosidace activity apart from the low basal activity was only a result of the presence of constitutive mutants and not caused by induced wild type cells.

To obtain an indication with respect to the degree of induction of the *lac*(con) mutant cells the specific β-galactosidace activity of *lac*(con) mutants was calculated according to equation 3 (neglecting the basal β-galactosidace activity of 2.5 Miller units). The results clearly suggest that the β-galactosidase activity per *lac*(con) mutant cell was changing during the experiment.

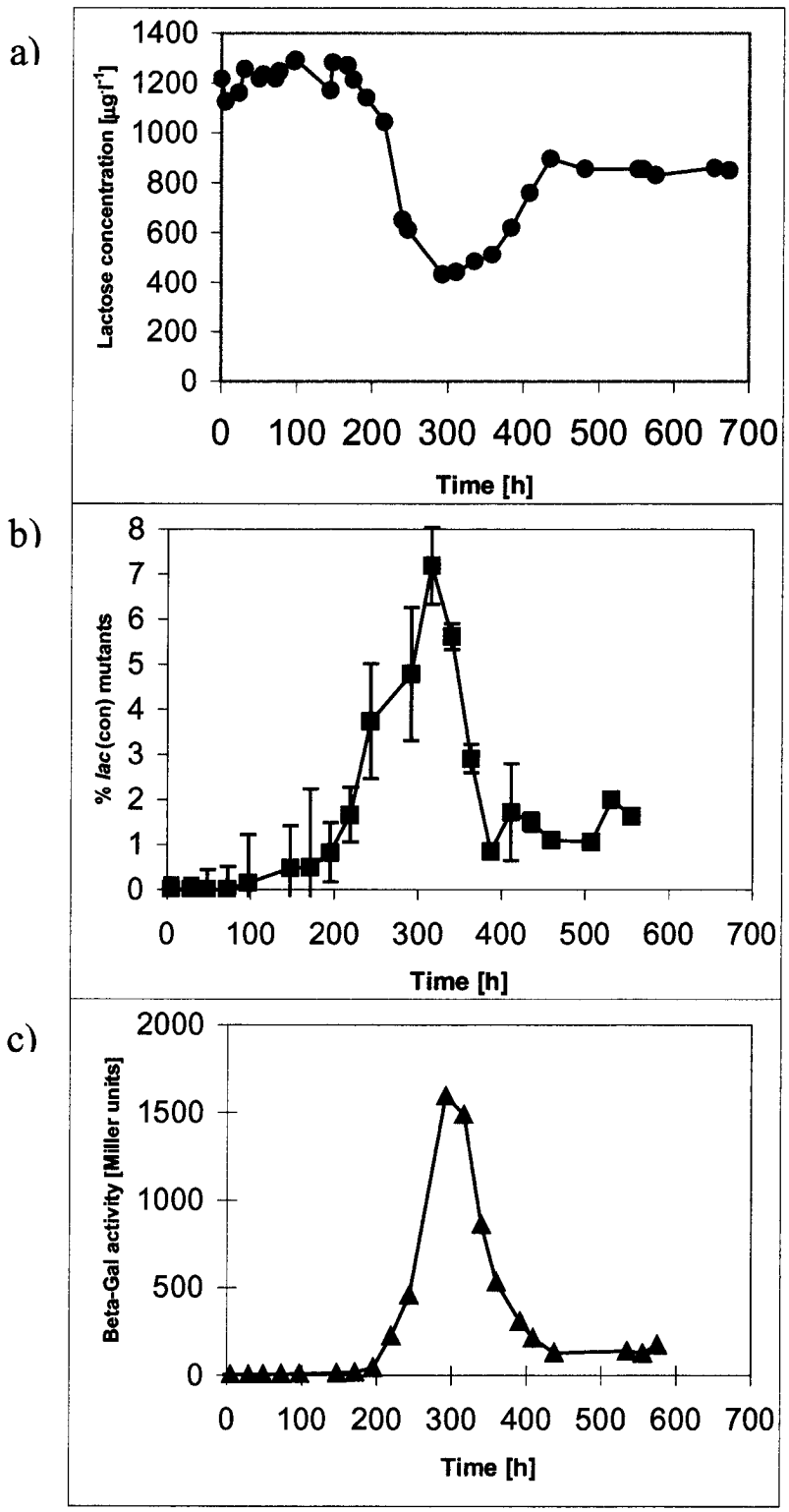

Fig. 3.1 Adaptation process of *E. coli* K12 to the glucose/lactose during growth in carbon-limited continuous culture at a dilution rate of 0.075 h^{-1} with 1.25 $mg \cdot l^{-1}$ lactose (1%) and 123.75 $mg \cdot l^{-1}$ glucose (99%). The residual lactose concentration in the culture (Fig. 3.1a), fraction of *lac*(con)mutants in the population (Fig. 3.1b) and the total β-galactosidase activity (Fig. 3.1c) was recorded.

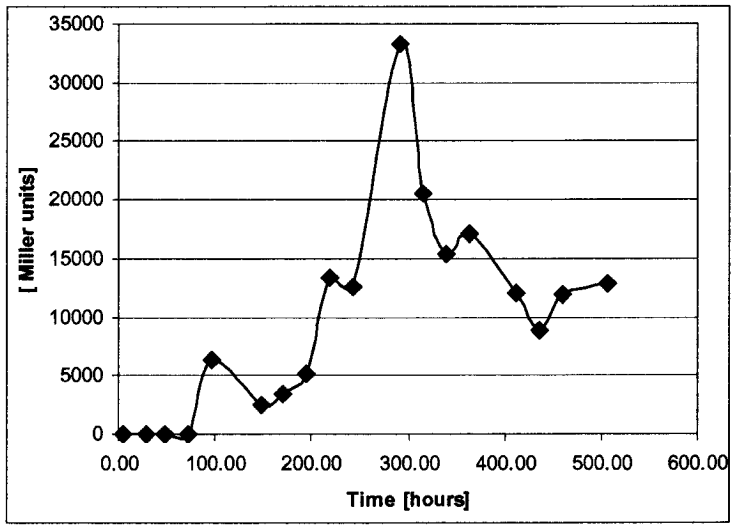

Fig. 3.2 The average specific β-galactosidase activity of *lac*(con) mutants during the selection of *E. coli* K12 in carbon-limited continuous culture at a dilution rate of 0.075 h^{-1} fed with a mixture of 1.25 $mg \cdot l^{-1}$ lactose (1%) and 123.75 $mg \cdot l^{-1}$ glucose (99%).

The specific β-galactosidase activity of a fully induced wild type culture is typically in the range of 1000 Miller units. However, *lac*(con) mutants are known to amplify the lac operon and can harbour up to 50 copies of this operon with a corresponding increase in the specific β-galactosidace activity (Andersson et al., 1998). Hence, the pattern of Miller units of *lac*(con) mutant suggests that the *lac*(con) mutants enriched in the culture contained transiently up to 35 copies of the lac-operon and this number stabilised at approximately 12 copies per *lac*(con) mutant cell.

Discussion and modelling

The gradual decrease of the concentration of the limiting carbon source during long-term cultivation in continuous culture has been documented in the literature for *E. coli* (Senn et al., 1994; Kovarova, 1997; Wick et al., 2001) and *Cytophaga johnsonae* (Höfle, 1983). For *E. coli* it was shown that this continuous decrease was due to a selection of mutants with improved affinity for the growth–limiting substrate (Ferenci, 1996; Tsen et al., 1996). Hence, also in the experiment reported here one would expect to find a continuously decreasing concentration of lactose and of glucose. Unfortunately due to analytical constraints it was only possible to determine the concentration of lactose but not of glucose.
During the first 300 hours of cultivation the experimentally observed lactose concentration
followed the expected pattern: First no lactose was consumed because its concentration was below the threshold needed for induction of the lactose operon (chapter 2), with time the spontaneously generated *lac*(con) mutants were selected and the fact that this fraction in the population increased to some 7% indicates that they not only lactose but at the same time successfully competed with the wild type for glucose. According to established mixed substrate kinetics cells able to utilise two or more growth-limiting substrates simultaneously have a competitive advantage because they are able to grow at reduced concentrations at the same dilution rate (Lendenmann et al., 1996). Hence, one expects that the lactose/glucose–utilising *lac*(con) mutant will outcompete wild type cells able to grow with glucose only. This was obviously not the case! Instead of taking over the culture completely (chapter 2) the fraction of *lac*(con) mutants decreased again and established itself at an apparent steady-state value of 1% of the total population. The most likely reason for the failure of the *lac*(con) mutant to take over the culture is the simultaneous appearance of non-*lac*(con) mutants with strongly improved affinity as reported for long-term glucose-limited cultures of *E. coli* (Ferenci, 1996; Wick et al., 2001). Such mutants are arising at a mutation rate of 10^{-7} per generation and the selected glucose-scavenging population has a 10-15 fold higher affinity to glucose compared to the wild type population (corresponding to a decrease in K_s from originally $800\mu g \cdot l^{-1}$ to $40\text{-}60\mu g \cdot l^{-1}$ (Wick et al., 2001). At the same time one has to assume that only the wild type but not

the *lac*(con) mutant can acquire this greatly improved affinity for glucose. This assumption seems justified since it has been reported that over-expressed lactose permease in *lac*(con) mutants negatively interferes with the glucose uptake system (Dykhuizen & Maxine, 1980). It appears that the two transporters compete for space on the cytoplasmic membrane. Hence, the initial success of the *lac*(con) mutant in competing for glucose with the wild type was hampered as soon as the high affinity glucose mutants appeared and it was forced back into its niche, i.e. to the utilisation of lactose. Nevertheless, the fact that the fraction of *lac*(con) mutants in the population was in order of 1-2 % by utilising approximately 0.5% of the total carbon in the feed indicates that they are still utilising a minor fraction of the supplied glucose (probably also in the order of 0.5% of the total glucose supplied).

Most interestingly, this segregation of the culture and selection process did apparently not lead to the best exploitation of the resources, i.e. the lowest possible lactose concentration. However, it can be argued whether or not the observed "steady-state" that established after some 400 hours of cultivation was really the final state of this evolving population or only a slowly changing "quasi-steady-state".

Simulation

Based on the consideration discussed above a model was set up (Fig. 3.1) and the model parameters used are listed in Table 3.1. The model essentially follows that used by

Wick for the simulation of the adaptation of *E. coli* to low glucose concentrations in continuous culture (Wick, 2002), but it was extended by integrating lactose utilisation by a *lac*(con) mutant. Parameterestimation suggested that *lac*(con) mutants from the wild type were formed at a mutation rate of $4.89 \cdot 10^{-9}$, whereas the mutants with higher glucose affinity appeared with a mutation rate of $5.88 \cdot 10^{-7}$ in succession as proposed by Wick (Wick, 2002). It was assumed that also *lac*(con)mutants can improve their glucose affinity, but only once and only to a $K_{sglucose}$ of 0.200 $mg \cdot l^{-1}$, due to the above mentioned transporter interference. For simplicity it was assumed that the *lac*(con) mutant did not evolve further but exhibited the same lactose utilisation kinetics (μ_{max}= 0.35 h^{-1}, K_s= 6.5 $mg \cdot l^{-1}$)

(Chapter 2) during the whole experiment, an assumption that is certainly open for discussion, considering the data given in Fig. 3.2.

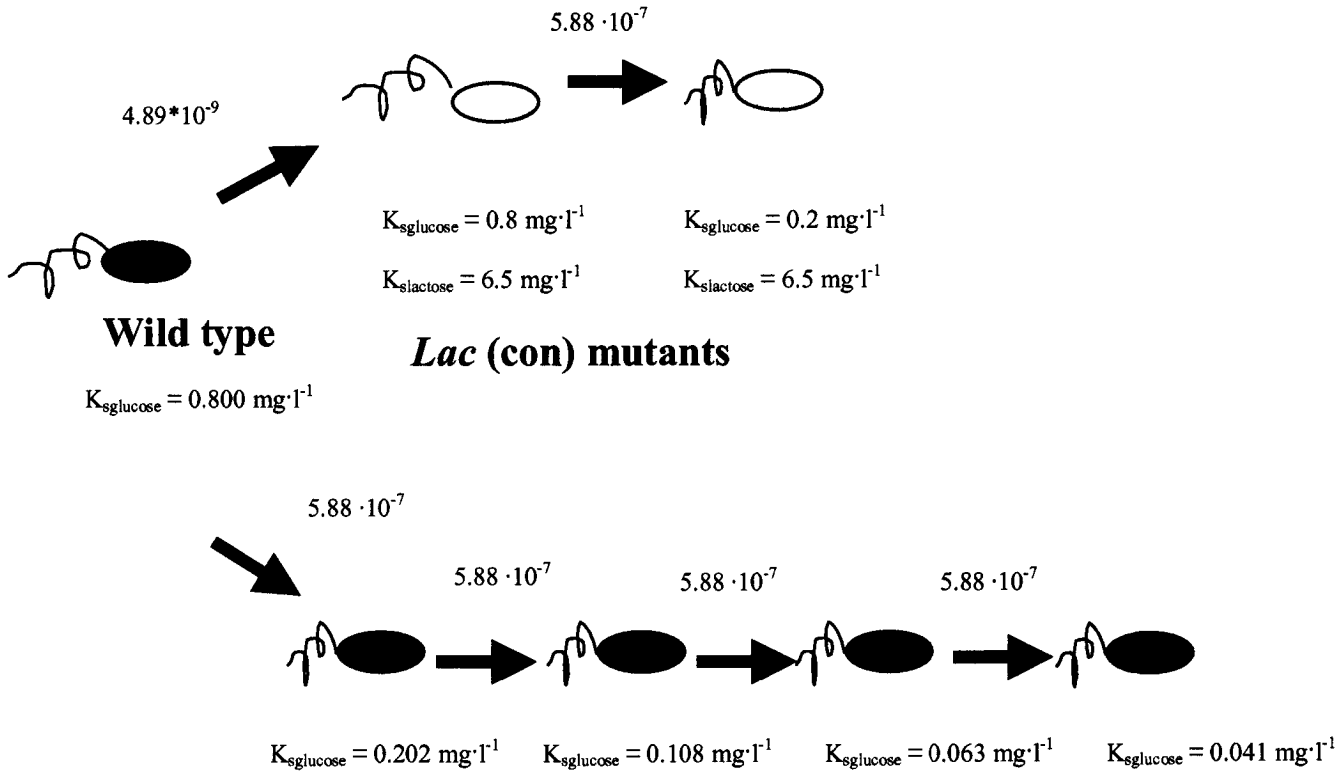

Fig. 3.3 Evolution and segregation of original *E. coli* K12 wild type population during long-term exposure to a mixture of glucose plus lactose (99%/1%) in carbon-limited continuous culture at a dilution rate of $0.075h^{-1}$. For other model parameters see Tab. 3.1.

For computer simulations the classical Monod kinetics extended with s_{min} were used to describe the single substrate utilisation of glucose by *E. coli* wild type and mutant strains (Kovarova & Egli, 1998).

$$\mu = \mu_{max} \frac{(s - s_{min})}{(K_s + (s - s_{min}))} \tag{4}$$

Growth of the *lac*(con) mutants with glucose and lactose simultaneously was mathematically described by simply adding up the specific growth rates supported by glucose and lactose as shown in equation (5).

$$\mu_{tot} = \mu_{maxgluc} \frac{(s_{gluc} - s_{min})}{(K_{sgluc} + (s_{gluc} - s_{min}))} + \mu_{maxlac} \frac{s_{lac}}{(K_{slac} + s_{lac})} \qquad \text{if } \mu_{tot} << \mu_{max} \tag{5}$$

Obviously this model can only be applied to cases where μ_{tot} is much lower than the specific maximum growth rates, otherwise the maximum specific growth rate of the culture will be exceeded as discussed previously (Lendenmann & Egli, 1997).
With a dilution rate of D= 0.075h^{-1} and the μ_{max} for glucose and lactose being 0.54 h^{-1} and 0.35 h^{-1}, respectively this condition is certainly fulfilled. The resulting concentration of glucose and lactose during growth of a population with both sugars was assumed to follow established steady-state mixed substrate kinetics for *E. coli* with sugar mixtures (equation 6) as described recently (Lendenmann et al., 1996; Lendenmann & Egli, 1997).

$$s_i = \mu_i \frac{K_{si}}{(\mu_{max} - \mu_i)} + s_{min} \tag{6}$$

i: glucose or lactose
$\mu_{tot} = \mu_{lactose} + \mu_{glucose} = D$

The numerical values of the parameters used for modelling (Table 3.1) were taken either from the literature (Wick, 2002) or from own experimental data. Only for the frequency of the generation *lac*(con) mutants a parameter estimation

was carried out in which the mutation rate was adjusted to fit the experimental data of the long-term continuous culture experiment presented in Fig. 3.1.

General parameter		
Name of parameter	Value used	Reference
$Yield_{glucose}$	0.45 g biomass·(g glucose)$^{-1}$	Chapter 1
$Yield_{lactose}$	0.45 g biomass·(g lactose)$^{-1}$	Chapter 1
Wild type		
$\mu_{maxglucose}$	0.63 h^{-1}	Wick, 2002
$K_{sglucose}$	0.800 $mg \cdot l^{-1}$	Wick, 2002
$s_{minglucose}$	0.012 $mg \cdot l^{-1}$	Wick, 2002
Parameters of *lac*(con) mutants		
$\mu_{maxglucose}$	0.63 h^{-1}	Wick, 2002
$K_{sglucose}$	0.800 $mg \cdot l^{-1}$	Wick, 2002
$s_{minglucose}$	0.012 $mg \cdot l^{-1}$	Wick, 2002
$\mu_{maxlactose}$	0.35 h^{-1}	Wick, 2002
$K_{slactose}$	6.5 $mg \cdot l^{-1}$	Chapter 1
Parameters of mutants with improved glucose affinity:		
$\mu_{maxglucose}$	0.67 h^{-1}	Wick, 2002
$K_{sglucosemut1}$	0.202 $mg \cdot l^{-1}$	Wick, 2002
$K_{sglucosemut2}$	0.108 $mg \cdot l^{-1}$	Wick, 2002
$K_{sglucosemut3}$	0.063 $mg \cdot l^{-1}$	Wick, 2002
$K_{sglucosemut4}$	0.041 $mg \cdot l^{-1}$	Wick, 2002
Mutation rates		
Wild type → *lac*(con) Mutant	$5.88 \cdot 10^{-7}$ (per cell replication)	Parameter estimation
Wild type → mutant with improved glucose affinity	$5.88 \cdot 10^{-7}$ (per cell replication)	Wick, 2002

Table 3.1 Parameters used for the mathematical simulation of the evolution of the *E. coli* wild type culture in glucose/lactose-limited chemostat culture.

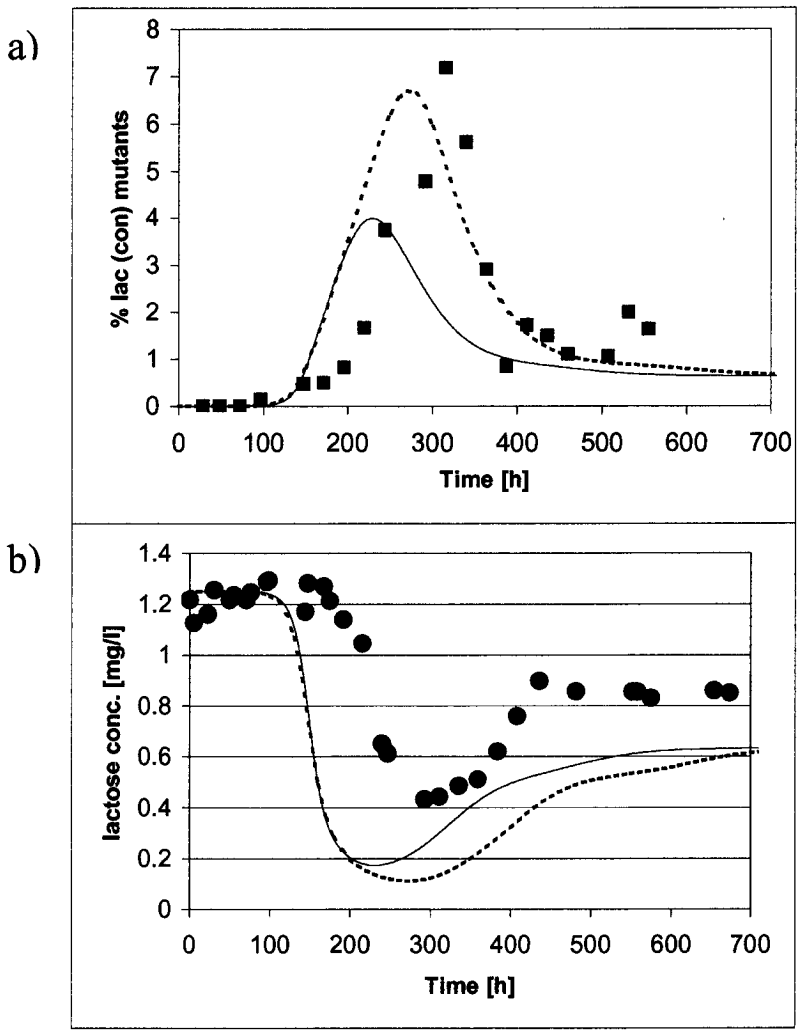

Fig. 3.4 Comparison of simulation (- fixed parameters (Tab 3.1), --- parameter estimation of mutation rate using a rate for mutation with higher glucose affinity of $5 \cdot 10^{-8}$ instead of $5.88 \cdot 10^{-7}$ per cell replication) and experimental data of the adaptation process with respect to the fraction of *lac*(con)mutants in the population (Fig. 3.4a) and the residual lactose concentration in the culture (Fig. 3.4b).

In Fig. 3.4 the model prediction and experimental data are compared. Taking into account that, except for the mutation rate for *lac*(con) mutants, all the parameter values had been adopted from the literature, the model nicely predicts all the trends observed in the experimental data.

Nevertheless, there are some significant deviations. For example, the predicted lactose concentration is lower than that observed experimentally. This may be explained by the observation that at low concentrations the cell wall markedly influences the uptake of lactose (Dean, 1989). Furthermore, the dynamics of

residual lactose concentration and the population change reflected by the proportion of *lac*(con) mutants in the culture, was slightly lagging behind the prediction. However, it was found that the outcome of the simulation was quite sensitive to slight changes in the model parameters. For example, a significantly improved fit for the *lac*(con) mutants population dynamics was obtained when decreasing the mutation rate for improved glucose affinity from $5.88 \cdot 10^{-7}$ to $5 \cdot 10^{-8}$ per cell replication. This value is still within the range reported in the literature for the mutation rate of 10^{-6} to 10^{-9} per cell replication (Novick & Szilard, 1950; Atwood et al., 1951; Kubitschek & Gustafson, 1964; Merrell, 1981; Chao & Cox, 1982; Birge, 1994; Tenaillon et al., 1999; Notley-McRobb & Ferenci, 2000). It should be pointed out that such subtle changes have been reported recently also for $K_{sglucose}$ at low the dilution rates $< 0.2\ h^{-1}$ resulting primarily from the increased expression of the "starvation" sigma factor rpos during slow growth (Wick, 2002). Considering all this, the deviation of the prediction when using parameters values from the literature is surprisingly small and therefore no further attempt was made to obtain a better fit of the model to the experimental data. In Fig. 3.5 the predicted succession of the different mutants during the experiment is shown. Interestingly, the predicted steady-state of the population with segregation into a *lac*(con) mutant and a mutant with improved glucose affinity was experimentally obviously already reached after some 400 hours of cultivation.

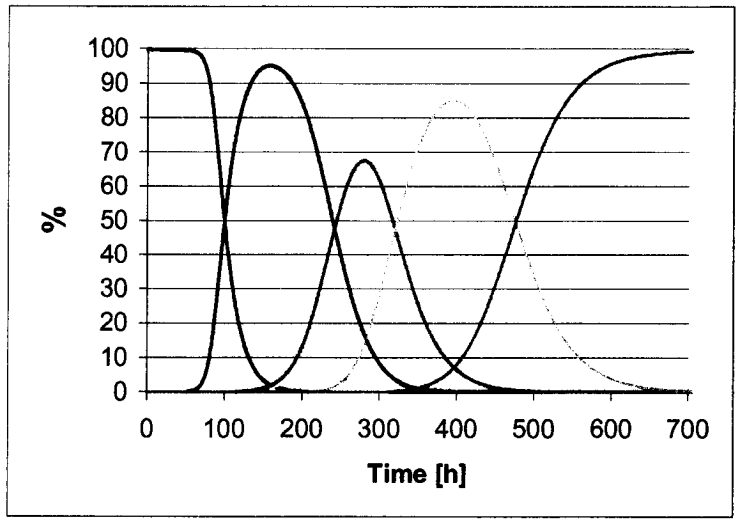

Fig. 3.5 Simulated population dynamics of all mutants with improved glucose affinity (original wild type (—), mutant1 (—), mutant2 (—), mutant3 ( ), mutant4 (—) and of the *lac*(con) mutant ( )) during evolution of *E. coli* K12 in carbon-limited continuous culture at a dilution rate of 0.075 h^{-1} with 1.25 $mg \cdot l^{-1}$ lactose (1%) and 125 $mg \cdot l^{-1}$ glucose (99%). The parameters in Table 3.1 were used for the simulation.

Conclusions

The experimental data presented confirms that for *E. coli* K12 a threshold concentration of approximately 2 $mg \cdot l^{-1}$ has to be exceeded for a successful induction of the lactose utilising system in the wild type. Extended exposure to 1.25 $mg \cdot l^{-1}$ of lactose was not sufficient for induction of the *lac* operon and utilisation of lactose was dependent on the appearance of lactose constitutive mutants. Surprisingly, the strategy of the *lac* (con) mutant to utilise glucose and lactose simultaneously was only transiently successful in the first phase of the experiment during which this mutant started to take over the culture. The price paid for being able to utilise lactose at such low concentrations was sufficient to outcompete the wild type *E. coli* strain.

However, it was obviously too high to compete with appearing mutants that exhibited an increased affinity for glucose. It seems that the wild type strain is not able improve both its lactose– and its glucose-scavenging ability at the same time. Hence, the logical outcome is the observed segregation of the population into two specialised mutant strains, each exploiting its own niche. Considering the physiological burden connected to the ability to utilise lactose at low concentrations it is not surprising that such mutants are not found among the natural isolates (Dykhuizen et al., 1987) and that utilisation of this disaccharide is tightly controlled and becomes only activated when a high threshold concentration is exceeded.

The fact that a model based on kinetic constants obtained from single substrate growth is able to reasonably well predict the outcome of the competition and evolution of the population reasonably well suggests that the presently used kinetic concepts of single and mixed substrate growth provide us with a good basis for the modelling of systems of increased complexity.

4. Competition for glucose in carbon-limited continuous culture between the enterobacterium *Escherichia coli* and *Chelatobacter heintzii,* an environmentally abundant bacterium

Hans Peter Füchslin, Christian Schneider and Thomas Egli

Abstract

The competition of an enterobacterium with a typical environmentally widely occuring bacterial strain under carbon limited conditions at low growth rates in a continuous culture system was studied using *Escherichia coli* strain ML30 (the enterobacterium) and *Chelatobacter heintzii* (the environmental strain) as a model system.

Initially the kinetic parameters μ_{max} and K_s of the two strains for growth with glucose were determined. In batch culture μ_{max} for both strains was determined after different precultivation conditions. In the case of *C. heintzii,* μ_{max} seems to be practically independent of precultivation conditions. *C. heintzii* inoculated from a glucose-limited continuous culture run at a dilution rate of 0.075 h^{-1} grew with a μ_{max} of 0.17 h^{-1} in batch culture with glucose as the only source of carbon and energy. After several transfers μ_{max} increased slightly to 0.18 h^{-1}. A different pattern was observed in the case of *E.coli.* Inoculated from a glucose-limited chemostat at D= 0.075 h^{-1} *E. coli* grew after lag phase of 3.5 hours with a μ_{max} of 0.54 h^{-1}. After 120 generations μ_{max} had increased to 0.80 h^{-1}. Furthermore, the maximum short-term glucose uptake rate measured in a glucose pulse experiment in continuous culture at D=0.075 h^{-1} was significant lower (factor 2.4) than the

q_{smax} calculated from batch experiments. In ^{14}C – labelled glucose uptake rate experiments a K_s for glucose of *C. heintzii* of 15 $\mu g \cdot l^{-1}$, and for *E. coli* of 35 $\mu g \cdot l^{-1}$ was determined.

The population dynamics of the mixed culture was determined using specific surface antibodies for *C. heintzii* and a specific 16S rRNA probe for *E. coli.C. heintzii* won clearly the competition for the carbon sources in a glucose-limited culture at the low dilution rates of 0.05 h^{-1} and 0.075 h^{-1}. Using the previously determined parameter values for K_s and μ_{max} the population dynamics during the competition experiment could be simulated by an extended form of Monod model that predicts a finite substrate concentration at zero growth rate (s_{min}).

The estimated s_{min} are for *E.coli* and *C.heintzii* were 22.3 $\mu g \cdot l^{-1}$ and 0 $\mu g \cdot l^{-1}$, respectively. The results of the mathematical simulation suggests that it is not the higher K_s – value, which is the main problem for the unsuccessful competition of *E. coli* at low residual glucose concentration but the existence of a significant s_{min}.

Introduction

The primary habitat of the enterobacterium *Escherichia coli* is considered to be the intestines of warm-blooded animals and men. However, enterobacteria are released daily from their hosts into the environment and it was estimated that surface, ground waters and soil, i.e. "the natural environment", are the second habitat for approximately half of the existing total *E. coli* population (Savageau, 1983). The two habitats differ enormously in the conditions they offer for microbial growth. Whereas in the intestine carbon/energy substrates are considered to be at least temporarily abundant (Koch, 1971) and the temperature is optimal (Savageau, 1983), microbial growth is limited in most environmental systems by the availability of carbon/energy sources and temperatures far from the optimum (Moriarty & Bell, 1993).

With *E. coli* being the standard indicator bacterium for assessing the microbial quality of drinking water and a potential fecal contamination (World Health Organization, 1996) there is considerable interest in understanding its survival and competitiveness under the conditions prevailing in the natural environment. In temperate climates and unpolluted sites it is assumed that most of the *E. coli* cells released into the environment are not able to survive for long but are dying quickly (Korhonen & Martikainen, 1991; Bogosian et al., 1996). However, it has been reported that under meso- and eutrophic conditions and tropical or subtropical temperatures between 15-45°C this enterobacterium can not only survive but also grow (Hazen & Toranzos, 1990; Tassoula, 1995; Solo-Gabriele et al., 1999). Such growth of *E. coli* in the environment conflicts with the use of *E. coli* as a general suitable indicator of water quality.

In our own laboratory we have recently observed that different *E. coli* strains were able to adapt to growth at low glucose concentrations. During long-term cultivation of the two strains *E. coli* ML30 and K12 in carbon-limited chemostat culture with glucose as the only carbon source a 10-20-fold improvement of the culture's affinity for glucose (K_s) from initially approximately 1 $mg \cdot l^{-1}$ to finally 50 $\mu g \cdot l^{-1}$ was reported (Lendenmann, 1994; Senn et al., 1994; Kovarova & Egli, 1998). On the other hand, temperatures away from the optimum were also found to affect growth kinetics by increasing the minimum glucose concentrations required for growth (s_{min}) (Kovarova et al., 1996a). However, due to analytical

limitations these kinetic parameters were determined at specific growth rates $>0.2h^{-1}$, which is considerably higher than those typically achieved by microbial cells in activated sludge or in the environment.

To investigate how these factors affect the competitiveness of *E. coli* for glucose during slow growth conditions we studied the competitive ability of a culture of *E. coli* ML30 adapted to low glucose concentrations in glucose-limited chemostat culture at 30°C at low dilution rates ($0.05\ h^{-1}$ and $0.075h^{-1}$). As a competition partner *Chelatobacter heintzii* was selected. This gram-negative bacterium seems to cope well under environmental growth conditions because it has been detected in high numbers not only in activated sludge (up to 1% of the total microbial population), but also in surface waters and soil (0.01-0.1% of total population) (Wilberg et al., 1992; Bally, 1994).

Materials and methods

Organisms and cultivation conditions

Organisms

Escherichia coli ML 30 (DSM 1329) and *Chelatobacter heintzii* (ATCC 29600) were used in all experiments.

Chemostat medium

The mineral medium contained, per litre: 275 mg NH_4Cl , 1.25 ml H_3PO_4 (85%) , 75 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot H_2O$, 35 mg KCl, 1.5 mg $FeCl_2$, 60 µg H_3BO_3, 100 µg $MnCl_2 \cdot 4H_2O$, 120 µg $CoCl_2 \cdot 6H_2O$, 70 µg $ZnCl_2$, 25 µg $NiCl_2 \cdot 6H_2O$, 15 µg $CuCl_2 \cdot 2H_2O$, 25 µg $Na_2MoO_4 \cdot 2H_2O$, 5.2 mg EDTA·$Na_4(H_2O)_4$.
The medium was sterilised by autoclaving at 120°C for one hour. After autoclaving
the mineral medium was supplemented of glucose as the only sources of carbon and energy using sterile disposable filters (0.22 µm, type GVWP, Millipore, Massachusetts, USA). The total glucose concentration was always 50 mg glucose carbon per litre. In addition, 0.25 ml of vitamin stock solution was added to 1 litre of medium by sterile filtration as described above. The stock solution contained per litre: pyrodoxin·HCl, 100 mg; 50 mg of each thiamine·HCl, riboflavin, nicotinic acid, D-Ca-pantothenic acid, *p*-amino benzoic acid, lipoic acid, nicotinamide and vitamin B_{12}; biotin 20 mg, and folic acid, 20 mg. Chemicals were purchased either from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

Batch medium

The mineral medium contained, per litre: 275 mg NH_4Cl , 1.25 ml H_3PO_4 (85%) , 75 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot H_2O$, 35 mg KCl, 1.5 mg $FeCl_2$, 60 µg H_3BO_3, 100 µg $MnCl_2 \cdot 4H_2O$, 120 µg $CoCl_2 \cdot 6H_2O$, 70 µg $ZnCl_2$, 25 µg $NiCl_2 \cdot 6H_2O$, 15 µg $CuCl_2 \cdot 2H_2O$, 25 µg $Na_2MoO_4 \cdot 2H_2O$, 5.2 mg EDTA·$Na_4(H_2O)_4$. After autoclaving the mineral medium was supplemented with glucose (≥98%, Fluka, Buchs, Switzerland) as the only source of carbon and energy by sterile filtration

using sterile disposable filters (0.22 μm, type GVWP, Millipore, Massachusetts, USA). The total glucose concentration was always 500 mg of carbon per litre. After heat sterilisation 100 ml of phosphate buffer ($Na_2HPO_4 \cdot 2H_2O$ / KH_2PO_4 0.56 M; with respect to phosphate; pH=7.5) was added by filtration to one litre of medium after cooling down to room temperature. Also added by filter sterilisation was 0.05 ml of vitamin stock solution per litre of medium. The vitamin stock solution contained per litre: pyrodoxin·HCl, 100 mg; 50 mg of each, thiamine·HCl, riboflavin, nicotinic acid, D-Ca-pantothenic acid, p-amino benzoic acid, lipoic acid, nicotinamide and vitamin B_{12}; biotin 20 mg, and folic acid 20 mg. Chemicals were purchased either from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

Cultivation

Continuous cultivation was performed in a 3.5 l bioreactor (MBR, Wetzikon, Switzerland) with working volume 2.8 litre equipped with both pH (7.50 ± 0.05) and temperature control (30±0.1 °C). For pH control a mixture of sterile 0.5 M NaOH/KOH was used. The impeller speed control was set to 1000 revolutions per minute and the oxygen saturation was > 90% air saturation. The bioreactors were regularly checked for wall growth to avoid artefacts as reported by (Pirt, 1975).

Analysis of mixed microbial populations

In competition experiments the composition of mixed population of *E. coli* and *C. heintzii* was followed using 5'end CY3-labelled 16S rRNA probe directed against *E. coli* in combination with cell surface antibodies specific for *C. heintzii*. The two methods were absolutely specific and no cross reaction was observed. For every sample two measurements were made one for the determination of the percentage part of *C. heintzii* and one for *E. coli*. Samples were first specifically stained by either specific antibodies or by 16S rRNA probe and subsequently all cells were stained by DAPI in order to determine the total bacterial number.

16S rRNA probe against *E. coli*

A specific 17-base-long 16S rRNA probe against *E. coli* was constructed (Wick et al., 2001) with the oligonucleotide sequence (ACTTTACTCCCTTCCTCCC).

Whole cell hybridisation was done according to the procedure described earlier by Manz and co-workers (Manz et al., 1992).

Specific surface antibodies against *C. heintzii*

The method used was adapted and modified from (Bally, 1994). All aqueous solutions were filtered before use (0.2 µm pore size) and all glassware was cleaned carefully with detergents, acid and alcohol to avoid any contamination before use. To approximately 2 ml of water culture sample one drop (20 µl) of formaldehyde (37%) was added. The sample was vortexed for one minute and kept in the refrigerator for 3 hours. Subsequently the samples were centrifuged (3 min, 13000 revolutions min^{-1}), the pelleted cells were washed three times with phosphate buffered saline (PBS; 14,4 $g \cdot l^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 3.2 $g \cdot l^{-1}$ $NH_2PO_4 \cdot 2H_2O$, 5.9 $g \cdot l^{-1}$ NaCl, pH 7.5) and finally resuspended in 2 ml of 50% ethanol / 50% PBS (vol/vol) and stored at -20°C.

For enumeration of *C. heinztii* the sandwich method as reported by Bally (Bally et al., 1994) was used. 30 µL of *Chelatobacter heintzii* ATCC 29600 strain-specific serum (polyclonal from rabbit (Bally, 1994)), diluted 100-times with PBS, were placed on a glass slide. Then an aliquot of prepared sample suspension (30 µl) was filtered through a black polycarbonate membrane filter (Nuclepore, Cambridge, USA, 0.4 µm pore size, 25 mm diameter) and the membrane filter was placed upon the serum. This allowed the serum to diffuse through the membrane filter without disturbing the homogeneous distribution of microbial cells on the filter surface. Slides were incubated for 30 minutes in a moist atmosphere at 25°C in the dark. Unbound antibodies were then removed by carefully rinsing the filter with 50 ml of PBS on a glass filtration fritte. The procedure was repeated using 30 µL of 50-times diluted fluoresceine isothiocyanate (FITC)-anti–rabbit–serum (F-0382, commercially available from SIGMA, St. Louis, USA).

4,6-diamidino-2-phenylindole (DAPI) staining

To determine the total cell number the cells fixed on the microscope filter stained with the specific antibody or 16S rRNA probe were covered with 30 µl of aqueous DAPI solution (10 µM) and incubated for 10 minutes. Subsequently the cells were washed with PBS in order to remove excess DAPI from the cells.

Microscopy and calculation of population composition

The black filter was placed on a glass slide with 20 µl of PBS. A cover slide suitable for fluorescent microscopy was put on it and covered with immersion oil (from Olympus, appropriate for fluorescence microscopy). The samples were examined within 10 hours using an Olympus BH-2 RFCA microscope (DplanApo 100UVPL objective, 10x eyepiece) equipped for epifluorescence microscopy.

The total (DAPI-stained) cell number was counted with UV-light (maximum at 360 nm) excitation, FITC-positive cells were enumerated using bluelight (495 nm) excitation and cells stained with the 16S rRNA labelled CY3-probe using redlight (350 nm) excitation with filter HQ-CY3 61241007 (AF Analysentechnik, Tübingen, Germany). Ten randomly chosen microscopic fields on filters with appropriate cell density (50-200) were counted at each wavelength.

The fraction of a specific strain given in % of the total population (P) was calculated using the following equation (1):

$$P(\%) = \frac{\sum_{z=1}^{10} \text{Number of specifically stained cells}}{\sum_{z=1}^{10} \text{DAPI stained cells}} * 100\% \qquad (1)$$

Glucose analysis

To avoid consumption of glucose during sampling the biomass was immediately separated from the culture liquid by filtration. Filtered samples were desalted by electrodialysis and subsequently the concentration of glucose was determined by HPLC separation, post-column reaction with alkaline copper(II) bisphenanthroline and amperometric detection. The method for sugar analysis has previously been published in detail (Lendenmann, 1994; Senn et al., 1994). Reliable glucose measurements were possible at concentrations > 10 $\mu g \cdot l^{-1}$.

Glucose uptake by whole cells

Uptake of ^{14}C-glucose was assayed by a rapid filtration method. Cells from the culture (2ml) were collected by centrifugation (13000 rpm, 2 minutes) and the supernatant was discarded. In order to keep disturbance of the cells at a minimum

the samples were diluted 250 - times relative to the original sample volume with filtered (0.2 μm pore size) spent chemostat cultivation medium.
The cell suspension in spent medium was incubated for 5 min at 30°C before ^{14}C-glucose was added to start the uptake experiment. As a function of time (15, 30, 45, 60, 90, 120, 150, 180 sec), aliquots were withdrawn from the assay mixture and immediately filtered through cellulose nitrate filters with a pore size of 0.25 μm (Sartorius AG, Göttingen, Germany). Without delay, the filters were washed with 20 ml PBS buffer containing 50 μM unlabelled glucose. Then, the filters were placed in scintillation vials and 3 ml of scintillation liquid (FilterCountTM, Packard Instrument B.V., Groningen, The Netherlands) was added. Finally, radioactivity incorporated was determined with a BETAmatic I liquid scintillation counter (Kontron Analytical, Zürich, Switzerland). All uptake rates reported are mean values of two or three independent transport assays. It is a known fact that uptake values can be compared relatively but that absolute values may vary considerably (Hunter & Kornberg, 1979).
It was observed earlier that the rates of incorporation of ^{14}C-glucose by *E. coli* were consistently lower than the rates at which the labelled glucose entered the cells. Obviously a large proportion of the glucose taken up was oxidised and hence lost as $^{14}CO_2$ (Hunter & Kornberg, 1979).
In our study, the uptake rate of *C. heintzii* corresponded well with the measured maximum specific growth rate. Converting the observed $q_{s(max)}$ (0.0070 mg substrate · mg dry weight $^{-1}$ · min^{-1}) into specific growth rate (equation 5) a μ_{max} of 0.16 h^{-1} was obtained which is similar to μ_{max} in batch culture (0.16 h^{-1}-0.18 h^{-1}).
In contrast in the case of *E. coli* $q_{s(max)}$ (0.005 min^{-1}) was very low. This is significant lower than the $q_{s(max)}$ measured in the pulse experiment (0.0144 ± 0.0005 min^{-1}).
In the previously mentioned study of Hunter & Kornberg a correction factor of 3.333 was reported. The $q_{s(max)}$ calculated from the ^{14}C – glucose uptake (0.00500 min^{-1}) multiplied by this factor (0.00500 min^{-1} · 3.333 = 0.01667 min^{-1}) is very close to the $q_{s(max)}$ determined in pulse experiment (0.0144 ± 0.0005 min^{-1}).

Dilution rate

The dilution rate, corresponding to the specific growth rate of the culture in chemostat is defined as

$$D = \frac{F}{V} \tag{2}$$

Theoretical wash-in and wash-out curves

Theoretical wash-out (3) and wash-in (4) curves were calculated by the following equation:

$$s(t) = s(0) \cdot e^{-Dt} \tag{3}$$

$$s(t) = s(0) \cdot (1 - e^{-Dt}) \tag{4}$$

Simulation software

The simulation and parameter estimation was carried out using AQUASIM, a program developed at our Institute (Reichert, 1998). AQUASIM allows estimating parameters by fitting calculated values (s_{obs}). This feature was used to estimate kinetical parameters, which resulted in the best fit of simulations and measurements. The program estimated the best fit by minimising χ^2 values ($\chi^2 = \Sigma((s_{obs} - s_{pred})/\sigma)^2$). For the estimation the standard deviation (σ) was set to 10% if not experimentally determined.

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Results

4.1 Determination of Monod kinetic parameters for glucose exhibited by *E. coli* and *C. heintzii*

Until today the empirical law of Monod (Monod, 1942) is the most widely used equation (1) to describe the kinetics of microbial growth. There is no consensus in the literature how the kinetic parameters should be measured and especially the values for K_s vary considerably for specific combinations of organism and substrate (Kovarova & Egli, 1998).

4.1.1 Determination of μ_{max} and q_s in batch culture and by pulsing glucose

In batch cultures the maximum specific growth rate, μ_{max}, for glucose of *E. coli* and *C. heintzii* was determined in the presence of 250 mg glucose·l^{-1}.

C. heintzii showed a relatively constant μ_{max} independent from the cultivation history. When this strain was precultivated in continuous culture at a dilution rate of 0.05 h^{-1} for at least ten days (26 generations) and was transferred into a batch culture they achieved a μ_{max} of 0.17 h^{-1} rigth away (after a short acceleration phase) and only a minor improvement was observed to a μ_{max} of 0.18 h^{-1} after 120 generations of cultivation in batch cultures.

A different behaviour was observed in the case of *E. coli*. When *E. coli*, precultivated in continuous culture at a dilution rate of 0.05 h^{-1}, was transferred in a batch culture with excess glucose only "linear" growth was recorded during the first 3.5 hours. After this lag phase exponential growth started and a μ_{max} of 0.54 h^{-1} was finally reached. It was observed that the μ_{max} of this population steadily increased over several generations of cultivation in batch cultures. After 62 generations a μ_{max} of 0.72 h^{-1} was observed and it further increased to 0.80 h^{-1} after 120 generations of batch cultivation. Fully "adapted" cultures of ML30 exhibited a μ_{max} of 0.92 h^{-1} (Lendenmann, 1994; Senn et al., 1994).

The actual physiological state and culture history have an impact on the available reaction potential (Grady et al., 1996). Although not able to immediately accellerate growth when challenged with excess carbon source cells when challenged with excess carbon source, cells can exhibit remarkably high carbon source consumption rates during slow growth. For sugars these rates are often

close to the maximum sugar consumption rate observed during exponential growth (Lendenmann & Egli, 1996). Therefore, we determined the q_{smax} for glucose of *E. coli* by pulsing excess glucose directly in to a culture of cells precultivated at D=0.075 h^{-1} for at least ten days (26 generations). The pulse of glucose injected was chosen such that initial glucose concentration was around 1 $mg \cdot l^{-1}$, which is well above the K_s of the culture for glucose (Wick, 2002). The medium supply was shut off immediately after the pulse and the decrease in glucose concentration was measured (Fig. 4.1). A linearly decrease of glucose was observed indicating that $K_s << s$. Hence, the relationship $q_s = q_{smax} \cdot s/(s+K_s)$ can be reduced to $q_s \approx q_{smax}$. The size of the pulse was such that it did not lead to a significant change in the total biomass concentration in the culture. Using this technique the maximum specific glucose consumption rate was determined by parameter estimation ($q_{s(max)}$= 0.0144 ± 0.0005 mg substrate·mg dry $weight^{-1} \cdot min^{-1}$). Converting this into specific growth rate (equation 5), assuming a constant yield of 0.39 mg biomass · mg $substrate^{-1}$, a μ_{max} of 0.34 ± 0.012 h^{-1} is obtained. We suggest that this value is the most appropriate to simulate growth kinetics under the slow growth conditions applied in our experiments (D=0.05 h^{-1} or 0.075 h^{-1}).

$$q_{s\,max} = \frac{\mu_{max}}{Y_{x/s}} \qquad (5)$$

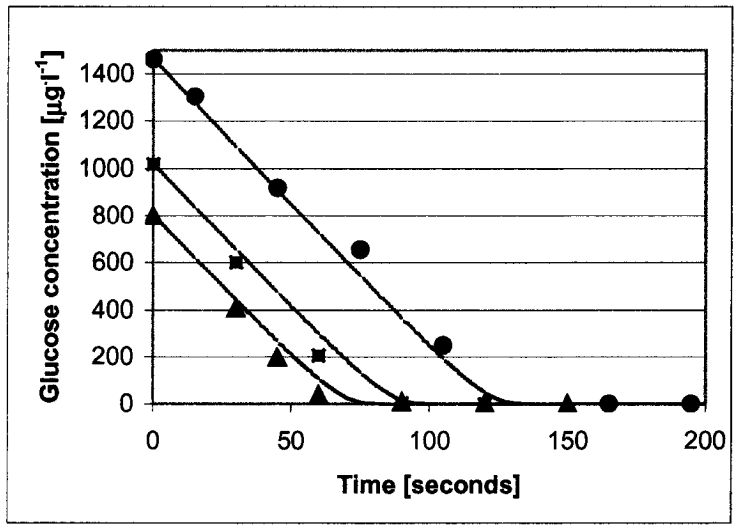

Fig. 4.1 Determination of q_{smax} of a culture of *E. coli* growing in a glucose limited chemostat at D=0.075 h^{-1}. The culture was pulsed with excess glucose and the resulting disappearance of glucose was monitored.

In summary, and as expected for a typical environmental K–strategist, *C. heintzii* exhibited a much lower μ_{max} than the enterobacterium *E. coli*.

4.1.2 Determination of K_s from glucose uptake rates

An alternative method frequently used to determine K_s consists of monitoring glucose uptake rates at different glucose concentrations using ^{14}C-labelled glucose.

For this cells of *E. coli* and *C. heintzii* were cultivated in continuous culture with glucose as the only carbon and energy source at a dilution rate of 0.075 h^{-1} at least for 10 days (26 genera-tions) before sample were taken. Particular attention was paid to the resuspension and dilution of the cells in order not to affect their uptake capacity and ability for glucose (see discussion in introduction). Therefore, samples were resuspended and if necessary diluted - with filtered (0.2 µm pore size), spent chemostat cultivation medium. The specific uptake rate as a function of glucose concentration is shown in Fig. 4.2 for *C. heintzii* and *E. coli,* respectively. The glucose uptake rate of cells of *C. heintzii* increased almost linearly up to a glucose concentration of 30 µg $\cdot l^{-1}$ (Fig. 4.2a). Although this pattern is closer to Blackman than Monod kinetics one can estimate a Monod K_s-constant to be in the order of 15 µg glucose$\cdot l^{-1}$.

The dependence of the glucose uptake rate of *E. coli* as a function of substrate concentration was similar to that observed for *C. heintzii* but the linear increase was even more pronounced and not as steep (Fig. 4.2b). The maximum specific uptake rate was reached only at approximately 70 µg glucose·l^{-1} and it was lower than that obtained from *C. heintzii*. The K_s of 35 µg glucose·l^{-1} deduced, corresponds well with the lowest K_s of 33 µg glucose·l^{-1} reported earlier for this strain (Kovarova et al., 1996a).

The discrepancy between the experimental data and the Monod kinetics was probably due to the slow dilution rate at which *E. coli* was growing. In our uptake experiments the available reaction potential of *E. coli* growing at D=0.075 h^{-1} was determined. *E. coli* has different glucose uptake systems of high and low affinity. In slowly growing *E. coli* cells especially high affinity uptake system are expressed and, therefore, the disagreement between the experimental data and the model proposed by Monod becomes understandable. As expected the affinity for glucose of the environmental strain *C. heintzii* was higher than that of *E. coli*.

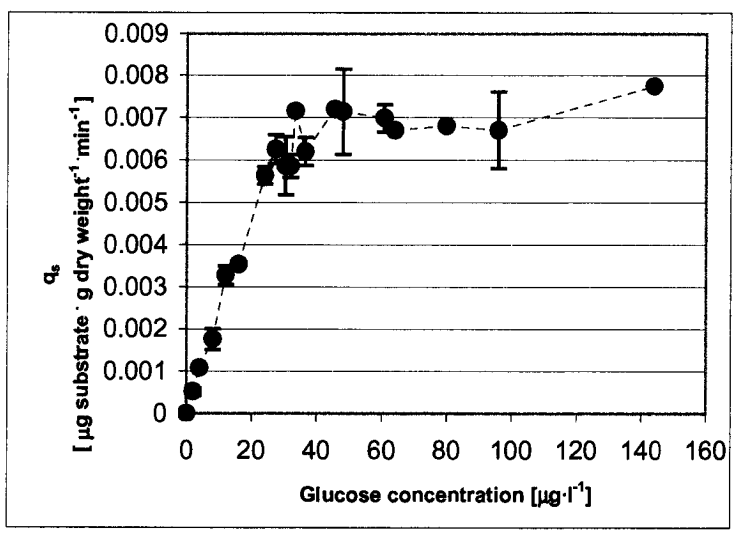

Fig. 4.2a Uptake rate of ^{14}C-glucose by *C. heintzii* at 30°C as a function of glucose concentration. The cells had been cultivated in glucose-limited continuous culture at D=0.075 h^{-1} and 30°C.

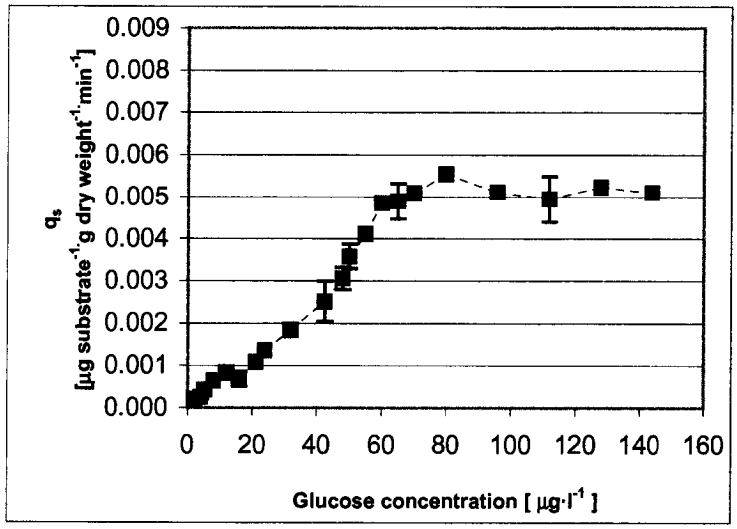

Fig. 4.2b Uptake rate of ^{14}C-glucose by *E.coli* at 30°C as a function of glucose concentration. The cells had been cultivated in glucose-limited continuous culture at D=0.075 h^{-1} and 30°C.

4.2 Competition for glucose in the chemostat at low dilution rates

E. coli and *C. heintzii* were first cultivated separately for ten days in glucose-limited continuous culture at a dilution rate of 0.05 h^{-1} in order to allow sufficient time for the adaptation of *both* strains to low glucose concentrations. After ten days 100 ml of culture liquid was removed from each chemostat and cross-inoculated to start the competition for glucose between the two strains. The composition of the population was monitored using eighter a specific 16S rRNA probe for *E. coli* or specific surface antibodies for *C. heintzii*. In both chemostat cultures *E. coli* was unable to compete with *C. heintzii* at D=0.05 h^{-1}. The results obtained for the mixed population for the *E. coli* chemostat inoculated with 100 ml *C. heintzii* are shown in Fig. 4.3. The total number of *E. coli* and *C. heintzii* added up to 97,9% ± 3.7 and corresponded well with the cell number determined by DAPI. A lag phase of 8 hours was observed before *C. heintzii* started to displace *E. coli*. After this lag the concentration of *E. coli* cells decreased slightly slower than predicted by a theoretical wash out-curve and after 120 hours of cultivation *E. coli* was virtually completely replaced by *C. heintzii*. The fact that the displacement of *E. coli* follows almost wash-out suggests that cells of *C. heintzii* exhibited a considerably better affinity for glucose than cells of *E. coli*. *E.*

coli cells inoculated into the *C. heintzii* chemostat were not able to compete and were washed out (data not shown). In both chemostat cultures at D=0.05 h^{-1}, the steady-state glucose concentration was below the detection limit of 10 $\mu g \cdot l^{-1}$ before and during the competition experiment. Higher growth rates favour in general opportunistic bacteria such as *E. coli* with high maximum specific growth rates and a low affinity. Therefore, the competition experiment was repeated at a slightly higher dilution rate of 0.075 h^{-1}. Because the quantification of *C. heintzii* was very reliable the fraction of *E. coli* was not determined in this second competition experiment.

Also at this dilution rate (Fig. 4.4) *C. heintzii* was able to out-compete *E. coli*, although not as quickly as at D=0.05h^{-1}. A short lag phase was observed also here before displacement of *E. coli* started and after 79 hours of cultivation cells of *C.heintzii* had taken over the culture.

The deviation from the theoretical wash-in curve was significantly more pronounced. Obviously *E. coli* is more competitive at this higher dilution rate. Once again it was not possible to reliably measure the steady-state residual concentration before and during the competition experiment because it was lower than 10 $\mu g \cdot l^{-1}$.

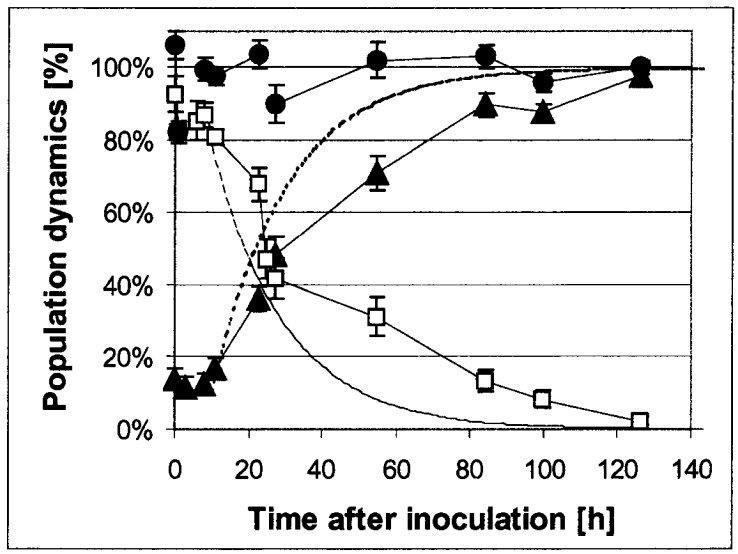

Fig. 4.3 Population dynamics of *E. coli* ( ) and *C. heintzii* (▲) during competition for glucose in a glucose-limited continuous culture at dilution rate of 0.05 h^{-1}. As a control, the sum of both strains is also given (●). For comparison the theoretical wash-in (---) and wash-out (—) curve are shown assuming a lag phase of 8 hours.

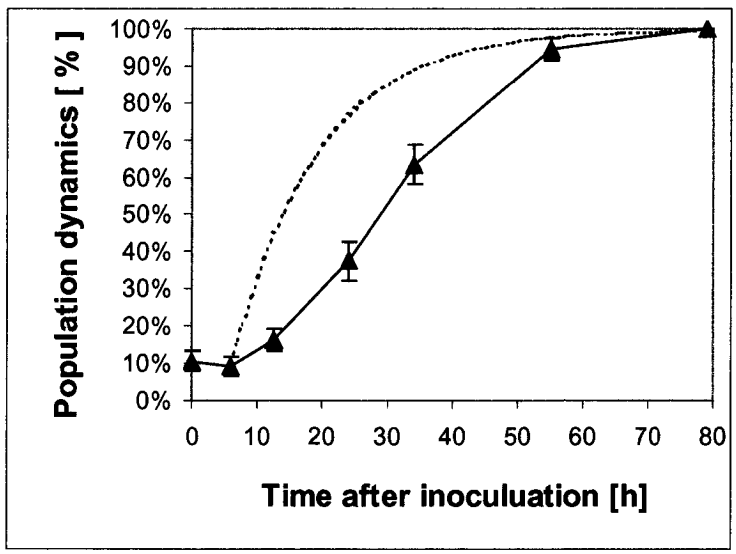

Fig. 4.4 Population dynamics *C. heintzii* (▲) during competition for glucose with *E. coli* in a glucose limited continuous culture at dilution rate of 0.075 h^{-1}. For comparison the theoretical wash-in curve is shown assuming a lag phase of 7 hours.

4.3 Competition for mixtures of glucose and lactose

In the two previously described experiments, *E. coli* was rapidly displaced by *C. heintzii* after a short lag phase. The data suggest that *E. coli* was virtually unable to withdraw glucose from the common pool. This raises the question whether or not interactions other than only competition for glucose took place. To test whether *C. heintzii* negatively affected growth of *E. coli* (for example by excreting an inhibitory metabolite) the mixed population was cultivated with different mixtures of glucose and lactose at a dilution rate of 0.075 h^{-1}. In contrast to glucose, which serves as a carbon source for both strains, only *E. coli* is able to consume lactose. Hence, we tested whether or not growth of *E. coli* with lactose was affected in the presence of *C. heintzii*.

The composition of the bacterial population and the residual lactose concentration were determined as a function of the glucose/lactose mixture in the feed medium (Fig. 4.5).

The fraction of *E. coli* cells increased linearly with increasing parts of lactose in the feed medium. The fact that the contribution of *E. coli* cells to the total population corresponded to the fraction of lactose in the feed indicates that *E. coli* was virtually totally excluded from the consumption of glucose by *C. heintzii*, and that it grew with lactose only. This supports the fact that *E. coli* has a substantial lower competitiveness for glucose than *C. heintzii*

During growth with all mixtures the steady-state concentration of lactose remained the same and was independent of the composition of the feed and the composition of the population (unfortunately in mixtures of glucose and lactose it was not possible to determine the residual concentration of glucose due to interference problem (see chapter 2)).

The unaffected lactose utilisation is a strong hint that growth of *E. coli* was not negatively influenced by the presence of *C. heintzii* suggesting that in the experiment shown in Fig. 4.3 and 4.4 competition for glucose was the only interaction between the two strains.

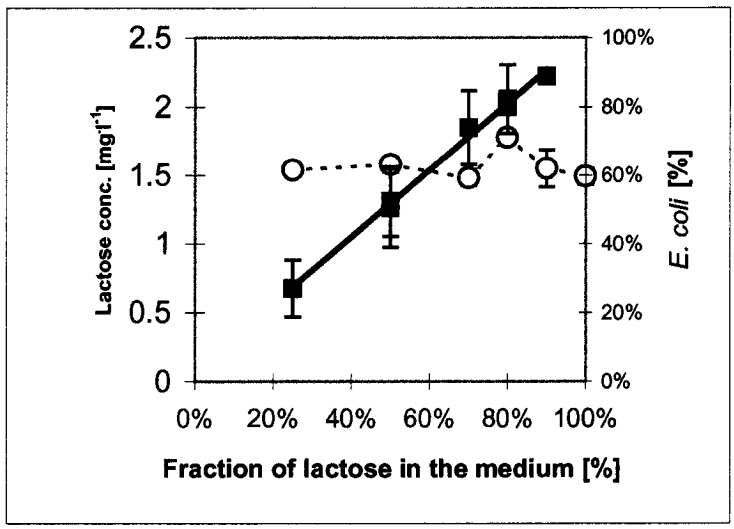

Fig. 4.5 Growth of mixed population of *C. heintzii* and *E. coli* with defined mixtures of lactose and glucose in a carbon-limited continuous culture at $D=0.075h^{-1}$ and 30°C. Percentage of *E. coli* in the bacterial population (■) and steady-state lactose concentration (○) is shown as a function of the percentage of lactose in the feed medium.

4.4 Simulation

We attempted to simulate the competition of the two bacterial strains performed in the chemostat using the kinetic parameters (K_s and μ_{max}) determined for *C. heintzii* and *E. coli*. Two different models were used for these simulations, namely the classical Monod model (equation 6) and the Monod model extended with s_{min} (equation 7).

$$\mu = \mu_{max} * \frac{s}{s + K_s} \tag{6}$$

$$\mu = \mu_{max} * \frac{(s - s_{min})}{(s - s_{min}) + K_s} \tag{7}$$

Furthermore, the following equations were used to describe the competition in the continuous culture:

$$\frac{dx_{E.coli}}{dt} = \mu_{E.coli} * x_{E.coli} - D * x_{E.coli} \tag{8}$$

$$\frac{dx_{C.\ heintzii}}{dt} = \mu_{C.\ heintzii} * x_{C.\ heintzii} - D * x_{C.\ heintzii} \tag{9}$$

$$\frac{ds}{dt} = D * s_0 - \frac{\mu_{E.coli} * x_{E.coli}}{Y_{x_{E.coli}/S}} - \frac{\mu_{C.heintzii} * x_{C.heintzii}}{Y_{x_{C.heintzii}/S}} - D * s \tag{10}$$

	μ_{max} (maximum specific growth rate)	K_s (affinity constant)	$Y_{X/S}$ (yield)	s_{min} predicted substrate concentration at D= 0 h^{-1}
E. coli	0.33 h^{-1}	35 $\mu g \cdot l^{-1}$	0.39	22.3 $\mu g \cdot l^{-1}$
C. heintzii	0.17 h^{-1}	15 $\mu g \cdot l^{-1}$	0.38	0 $\mu g \cdot l^{-1}$

Table 4.1 Model parameters and their values used for the simulation of growth and competition for glucose in carbon-limited continuous cultures at 30°C.

When the classical Monod kinetics with the experimentally determined parameters (Table 4.1) are applied for a dilution rate of 0.05 h^{-1} the model predicts a virtually stable culture of the two strains, whereas at D=0.075 h^{-1} it predicts that *E. coli* would win the competition for glucose. This is in contradiction to the experimental outcome and the difference can not simply be explained by inaccuracies in the determination of the kinetic parameters. It is more likely that the model does not fit the data. Especially at low growth rates maintenance energy can play a significant role and is known to affect competition and kinetics.

For this reason, the Monod model extended with the term s_{min} (equation 7), was tested using same values of the kinetic parameters as in the previous simulation. As it was not possible to measure s_{min} directly, the value of s_{min} was adjusted such that simulation and experimental data were in best agreement for both competition experiments (D=0.05 h^{-1} and D=0.075 h^{-1}). The values obtained for s_{min} determined by parameter estimation were 22.3 $\mu g \cdot l^{-1}$ for *E. coli* and zero for *C.heintzii*. The value of 22.3 $\mu g \cdot l^{-1}$ is very similar to the s_{min} of 18 $\mu g \cdot l^{-1} \pm 2$ at 30°C, reported by Kovarova (Kovarova et al., 1996a).

The simulation using the extended Monod model and the fit with the experimental data are shown in Fig. 4.6 and 4.7.

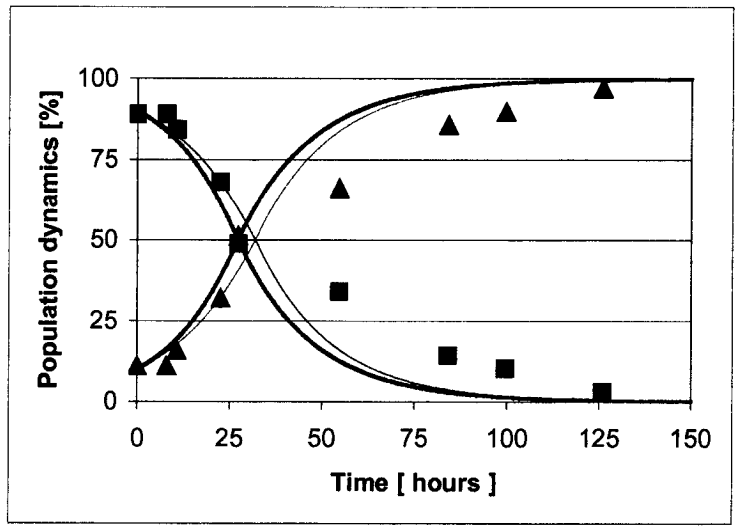

Fig. 4.6 Prediction of competition for glucose for a mixed culture of *C. heintzii* (▲) and *E. coli* (■) in a glucose-limited continuous culture at a dilution rate of 0.05 h^{-1} using the fits for the different s_{min} are shown, namely for s_{min}=18.0 $\mu g \cdot l^{-1}$ (—) from (Kovarova, 1996b) and for s_{min}= 22.3 $\mu g \cdot l^{-1}$ (—) , a value obtained by parameter estimation.

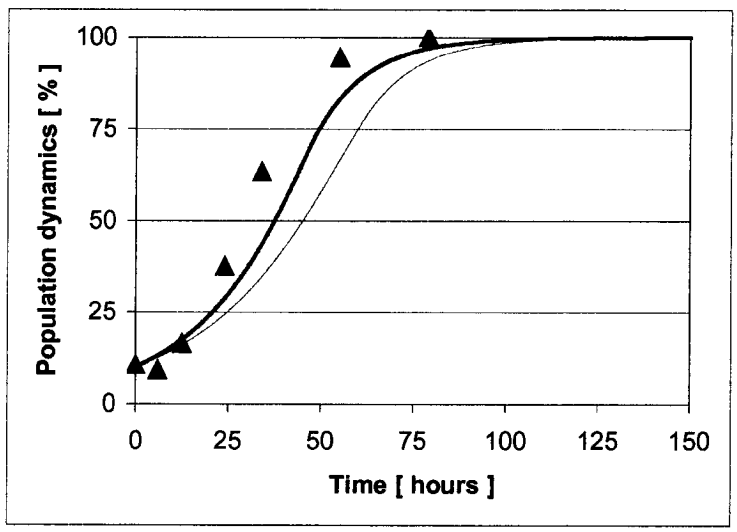

Fig. 4.7 Prediction of competition for glucose for a mixed culture of *C. heintzii* (▲) and *E. coli* in a glucose-limited continuous culture at a dilution rate of 0.075 h^{-1} using the fits for the different s_{min} are shown, namely for s_{min}=18.0 $\mu g \cdot l^{-1}$ (—) from (Kovarova, 1996b) and for s_{min}= 22.3 $\mu g \cdot l^{-1}$ (—) , a value obtained by parameter estimation.

Discussion

As expected, the environmentally successful bacterium *C. heintzii* clearly won the competition for glucose against the enterobactierum *E. coli* at low dilution rates tested (D=0.05 h^{-1} and 0.075 h^{-1}). Due to only moderate difference in the K_s exhibited by *C. heintzii* and *E. coli* it was only possible to simulate the fast displacement of *E. coli* in the competition experiment only by using the Monod kinetics extended with s_{min}. For the simulation experimentally determined kinetic parameters (μ_{max}, K_s) were successfully used whereas the values for s_{min} had to be determined by parameter estimation. The value for s_{min} for *E. coli* of 22.3 $\mu g \cdot l^{-1}$ is very similar to the value reported earlier (18 $\mu g \cdot l^{-1} \pm 2$) for growth of this bacterium with glucose at 30 ^{0}C (Kovarova et al., 1996a). The existence of a cellular maintenance energy requirement can be easily explained by thermodynamic reasoning. The cell has to maintain concentration gradients and has to restore continuously its cellular components ageing by oxidative stress, denaturation etc. (Dawes, 1984). The consequence is the requirement of a finite concentration or flux of an energy (carbon) substrate at zero growth rate (s_{min}) (Kovarova & Egli, 1998). A possible explanation for the significant s_{min} of *E. coli* could be that *E. coli* is adapted to a pulsed substrate supply as and it is able to use high glucose availability by high growth rates. One can speculate that maintaining a metabolism that is capable of a high maximum growth rate needs more energy than the metabolism that is adapted to low growth rates as the one of *C. heintzii.*

The specific μ_{max} for glucose of *C. heintzii* was virtually independent of the actual growth conditions. Hence, *C. heintzii* may have only one glucose transport system with low maximal uptake rate but high glucose affinity. In contrast, *E. coli* is exposed during its life cycle to different glucose concentrations and has therefore different uptake systems for glucose with different affinities (Ferenci, 1999). Consequently, the μ_{max} and also $q_{s(max)}$ is noticeable dependent of the culture history and the actual growth conditions of the *E. coli* cell (Grady et al., 1996; Ferenci, 1999).

Also after an adaptation time of 26 generations *E. coli* exhibited a lower glucose affinity (K_s= 35 $\mu g \cdot l^{-1}$) than *C. heintzii* (K_s= 15 $\mu g \cdot l^{-1}$). Our data were very similar

to the K_s value for glucose reported for *E.coli* (33 $\mu g \cdot l^{-1}$) adapted to low glucose concentration in continuous culture (Kovarova, 1997; Wick et al., 2001).
The K_s–value of *C. heintzii* of 15 $\mu g \cdot l^{-1}$ for glucose is in the midfield of glucose K_s values reported in the literature for bacterial strains, ranging from a lake isolate (5 $\mu g \cdot l^{-1}$) (Hobbie & Wright, 1965) to the widespread *Aeromonas hydrophila* (33 $\mu g \cdot l^{-1}$) (Van der Kooij et al., 1980). From our simulation results it can be concluded that it is not the high K_s – value that is the main problem for the competition at slow growth rates for *E. coli* but the existence of a significant s_{min}.
In contrast to our experiments bacteria will be exposed in the environment to varying concentration of different substrates (Münster, 1993; Egli, 1995) and changing physiochemical conditions as pH, temperature and so on. It will be interesting whether or not *E. coli* is able to coexist under fluctuating substrate supply. This question will be addressed in the next chapter.
Of interest is also the observation of the existence of significant s_{min} exhibited by *E. coli* can this be generalised for enterobacteria or copiotrophes and, how is it possible for *C. heintzii* to minimise their s_{min} to such a low level? Such information is crucial for the better understanding of microbial degradation and growth processes in the environment.

5. Fluctuation of glucose supply enables the "opportunist" *Escherichia coli* to co-exist in cultures with the "gleaner" *Chelatobacter heintzii*

Hans Peter Füchslin and Thomas Egli

Abstract

Under natural growth conditions fluctuating supply of nutrients is the rule rather the exception and experience shows that under such conditions the coexistence of strains with differing kinetic properties is possible. For the field of microbiological testing of water and drinking water quality it is of considerable interest to know whether or not Enterobacteria can survive and grow under environmental conditions.

Therefore, a simulation study based on the experimentally determined growth kinetic parameters was performed to assess the ability of the "copiotrophic" bacterium of *Escherichia coli* to grow and compete with *Chelatobacter heintzii*, an environmentally ubiquitous and "oligotrophic" bacterium, at glucose concentrations as they are observed to fluctuate in natural surface waters. In addition to changing glucose concentrations also the impact of temperature on the competition between *C. heintzii* and *E. coli* was studied.

The analysis suggests that *E. coli* can survive and even growth in the presence *of C. heintzii* under such conditions when the temperature is higher than 29°C. The main competitive disadvantages of *E. coli* under environmental growth conditions are its low substrate affinity, its relatively high maintenance energy requirements (s_{min}), and the high optimum temperature for growth.

Introduction

In flow through systems, such as the continuous culture, complete competitors can not coexist when competing for a single limiting nutrient under ideal conditions (Gause, 1934; Hardin, 1960; Gottschal, 1993). Consequently, supposing ideal conditions, the diversity of a mixed microbial population should be directly related to the number of growth limiting substrates that are available in a system, and for each substrate there would be one "winner". Although a wide range of different carbon sources exists in natural aquatic systems (Münster, 1993) this would not explain the multitude of different microbial strains and the complex composition of microbial consortia.

Hutchinson was the first to propose that temporal variation may be an explanation for the observed diversity of competing species in physical habitats (Hutchinson, 1961). Besides fluctuating physicochemical growth conditions (temperature, pH, ion strength, O_2 concentration, osmolarity etc.) the fluctuating supply of growth substrates has been proposed to support the coexistence of different species in the same environment (Frederickson & Stephanopoulos, 1981) and to explain the high number of different microbial species in the environment.

Following this up for the case of a pure and simple competition (Frederickson & Stephanopoulos, 1981) between two species in a continuous culture, some theoretical studies have predicted that periodic fluctuations of the chemostat dilution rate (Grenney et al., 1973; Stephanopoulos et al., 1979) or of the limiting nutrient concentration in the medium feed (Hsu, 1980; Smith, 1981; Hale & Somolinos, 1983) allows the two microbial strains to coexist in a stable-state of periodic oscillations.

In the field of microbiological water quality testing, where throughout the world *E. coli* is used as an indicator of fecal contamination (WHO), the survival and possible growth of enterobac-teria and their coexistence with indigenous environmental flora in surface waters is of special interest.

The primary habitat of the enterobacterium *Escherichia coli* is the intestine of warm-blooded animals and men (Savageau, 1983). However, day by day, myriads of enterobacteria are released from their hosts into the environment (Stephen & Cummings, 1980; Lee, 1985), and the two habitats differ enormously

in the conditions they offer for microbial growth. Whereas in the intestine carbon/energy substrates are usually abundant and the temperature is optimal, microbial growth is limited in most environmental systems by the scarce availability of carbon/energy sources and temperatures far from the optimum (Egli, 1995; Morita, 1997).

E. coli can be characterised as a r-type species with high production rate and average affinity for glucose (Atlas & Bartha, 1981). Hence, it is viewed as an opportunist waiting for high substrate pulses and using its advantage of a high specific growth rate to consume the initial flush of substrates ahead of its competitors. It is generally assumed that this enterobacterium is not able to survive and grow under normal oligotrophic environmental conditions in the Northern hemisphere. However, in the tropical hemisphere *E. coli* is known to be a part of the microbial population in surface waters (Hazen & Toranzos, 1990; Solo-Gabriele et al., 1999).

Such results challenge the use of *E. coli* as a suitable indicator of water quality throughout the world. They also suggest that pathogenic enterobacteria might grow in certain environments and present a health risk.

The study was initiated because it was observed in our laboratory that extended growth of *E. coli* at low glucose concentrations allows this enterobacterium to adapt and to develop an improved affinity for this sugar (Lendenmann, 1994; Kovarova & Egli, 1998; Wick et al., 2001). During long-term cultivation of the two strains *E. coli* ML30 and K12 in carbon-limited chemostat culture with glucose as the only carbon source a 20-30-fold improvement of the culture's affinity for glucose (K_s) from initially approximately 1 $mg \cdot l^{-1}$ to finally 35 $\mu g \cdot l^{-1}$ was reported. In this work the ability of *E. coli* to compete with a bacterial strain isolated from the environment is investigated under laboratory conditions.

As a sparring partner for competition the gram-negative bacterium *Chelatobacter heintzii* was chosen because it seems to cope very well under environmental growth conditions and it has been detected in high numbers not only in activated sludge (up to 1% of the total microbial population), but also in surface waters and soil where it can account for 0.01-0.1% of total population (Wilberg et al., 1992; Bally, 1994). Obviously, *C. heintzii*, a so-called gleaner, used to grow at very low substrate concentrations with high affinity uptake systems.

In this modelling study the general conditions necessary for coexistence of two species with different kinetical parameters (gleaner and opportunist) under fluctuating substrate supply are first investigated. Then the ability was assessed for *E. coli* cells to grow and compete with *C. heintzii* under fluctuating conditions of glucose supply as they occur in natural surface water. In addition, the crucial impact of temperature on the competition between *C. heinzii* and *E. coli* was studied.

Materials and methods

Simulation software

The simulation and parameter estimation was carried out using AQUASIM, a program developed at our institute (Reichert, 1998). AQUASIM allows estimating parameters by fitting calculated values (s_{obs}). This feature was used to estimate kinetical parameters, which resulted in the best fit of simulations and measurements. The program estimated the best fit by minimising χ^2 values ($\chi^2=\sum((s_{obs}-s_{pred})/\sigma)^2$). For the estimation the standard deviation (σ) was set to 10% if not experimentally determined.

Batch medium

The mineral medium contained, per litre: 275 mg NH_4Cl , 1.25 ml H_3PO_4 (85%) , 75 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot H_2O$, 35 mg KCl, 1.5 mg $FeCl_2$, 60 µg H_3BO_3, 100 µg $MnCl_2 \cdot 4H_2O$, 120 µg $CoCl_2 \cdot 6H_2O$, 70 µg $ZnCl_2$, 25 µg $NiCl_2 \cdot 6H_2O$, 15 µg $CuCl_2 \cdot 2H_2O$, 25 µg $Na_2MoO_4 \cdot 2H_2O$, 5.2 mg $EDTA \cdot Na_4(H_2O)_4$. The mineral medium was supplemented with glucose (≥98%, Fluka, Buchs, Switzerland) as the only source of carbon and energy. The total glucose concentration was always 500 mg of carbon per litre. After heat sterilisation 100 ml of phosphate buffer ($Na_2HPO_4 \cdot 2H_2O$ / KH_2PO_4 0.56 M; with respect to phosphate; pH=7.5) was added by filtration to one litre of medium after cooling down to room temperature using sterile disposable filters (0.22 µm, type GVWP, Millipore, Massachusetts, USA). Also added by filter sterilisation was 0.05 ml of vitamin stock solution per litre of medium. The vitamin stock solution contained per litre: pyrodoxin·HCl, 100 mg; 50 mg of each, thiamine·HCl, riboflavin, nicotinic acid, D-Ca-pantothenic acid, p-amino benzoic acid, lipoic acid, nicotinamide and vitamin B_{12}; biotin 20 mg, and folic acid 20 mg. Chemicals were purchased either from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

5.1 General aspects for coexistence under a regime of fluctuating substrate supply

In previous work (chapter 2) we have observed that *C. heintzii* reproducibly displaced *E. coli* when competing for glucose in carbon-limited continuous culture at low dilution rates. The kinetic constants for the growth of the two bacterial strains, either experimentally determined or collected from the literature, (Table 4.1) clearly demonstrate the "opportunist" properties of *E. coli* with a higher specific maximum growth rate but a lower glucose affinity compared to the gleaner *C. heintzii*. Furthermore, a significant difference between the two strains is the existence of a distinct minimum concentration for growth for *E. coli*, whereas s_{min} was undetectable low for *C. heintzii*.

	μ_{max}	K_s	s_{min}	$Y_{X/S}$
E. coli	0.33 h^{-1}	35 $\mu g \cdot l^{-1}$	22.30 $\mu g \cdot l^{-1}$	0.39
C. heintzii	0.17 h^{-1}	15 $\mu g \cdot l^{-1}$	0 $\mu g \cdot l^{-1}$	0.38

Table 5.1 Kinetic constants experimentally determined for the growth of *E. coli* and *C. heintzii* with glucose at 30 °C data from Chapter 4.

Two different kinetic models describe the growth of the two strains well, namely the classical Monod model (equation 1) for *C. heintzii*, and the Monod model extended with s_{min} (equation 2) for *E. coli*.

$$\mu_{Ch} = \mu_{max_{Ch}} * \frac{s}{K_{s_{Ch}} + s} \qquad (1)$$

$$\mu_{Ec} = \mu_{max_{Ec}} * \frac{(s - s_{min})}{K_{s_{Ec}} + (s - s_{min})} \qquad (2)$$

It has been predicted (Gottschal, 1993) that it will be possible for a r-strategist and a k-strategist to coexist in a continuous culture system on conditions that the supply of substrate in the feed alternates (this is in contrast to the traditional continuous cultivation with a constant glucose feed concentration). To test under which conditions *E. coli* and *C. heintzii* would be able to coexist using the experimentally determined kinetic constants a number of competition experiments were simulated with differently alternating glucose supply using the modelling software Aquasim.

As a model a continuous culture at the dilution rate of 0.075 h^{-1} was chosen where the dilution rate stands for a general death rate, including cell autolysis, grazing of bacterial cells by protozoa, and dilution. Coexistence was found when, in 24 hour intervals the glucose was supplied at the following pattern: In the first 6 hours the glucose concentration in the feed medium was 3 $mg \cdot l^{-1}$ (high concentration phase) whereas in the following 16 hours it was reduced to 0.3 $mg \cdot l^{-1}$ (low concentration phase). In order to elucidate the factors which enable the coexistence, the 24 hour periods was analysed in detail (Fig. 5.1). The changing glucose concentration in the medium (Fig. 5.1a) leads to an alternating residual glucose concentration in the chemostat (Fig. 5.1b). During low glucose supply the K-strategist *C. heintzii* was utilising virtually all of the glucose because of its higher substrate affinity and the high s_{min} of *E. coli* resulted in the wash-out of the enterobacterium from the culture. The change from the low to high feed glucose concentration resulted in an accumulation of glucose due to the low growth rate and low biomass of *C. heintzii*. This allowed *E. coli* to recover and regrow to a significant population size and to avoid the complete wash-out during the subsequent low glucose period. In such a way the populations of *E. coli* and *C. heintzii* are oscillating but can coexist for extended periods of time (Fig. 5.2).

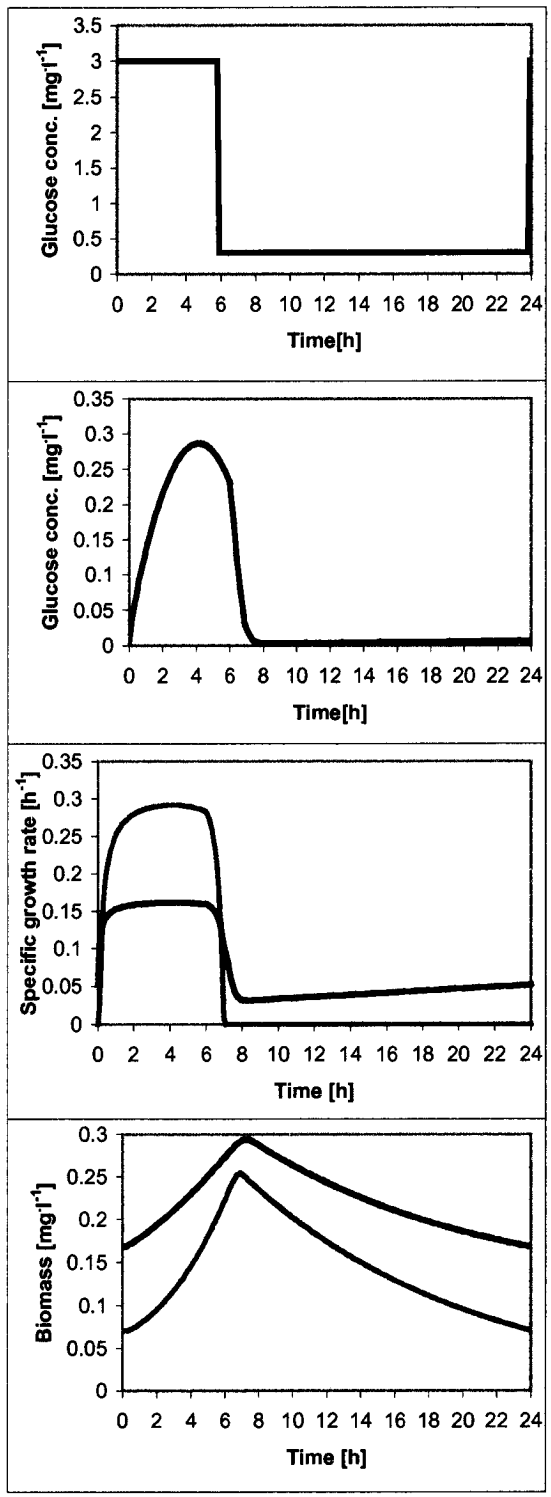

Fig. 5.1 Simulation of the competition between *C. heintzii* (—) and *E. coli* (—) for glucose in continuous culture at the dilution rate of 0.075 h^{-1} and fluctuating periodic substrate supply during a 24 hour period. The culture is in a oscillating steady-state.
a) Concentration of glucose in the feed medium
b) Glucose residual concentration in the culture
c) Specific growth rate of *E. coli* and *C. heintzii*
d) Biomass dynamics of *E. coli* and *C. heintzii*

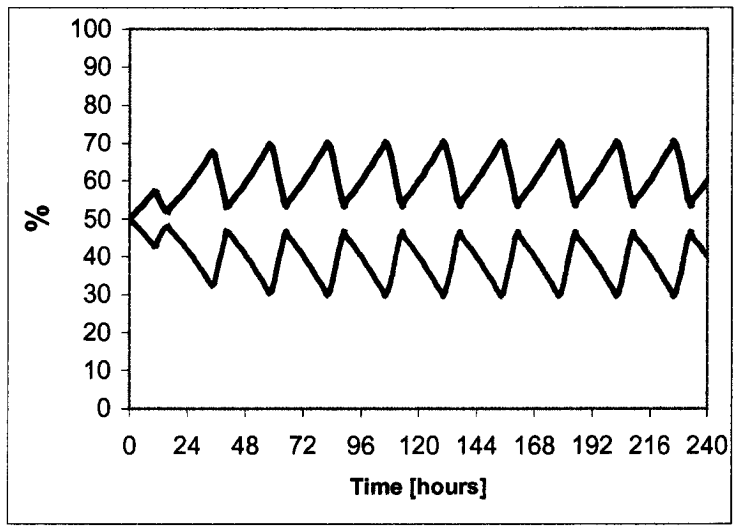

Fig. 5.2 Fluctuating supply leads to oscillating population dynamics of *C.heintzii* (—) and *E. coli* (—) and coexistence. The substrate supply oscillates as described in Fig. 5.1 but the population dynamics are depicted here for an extended time period.

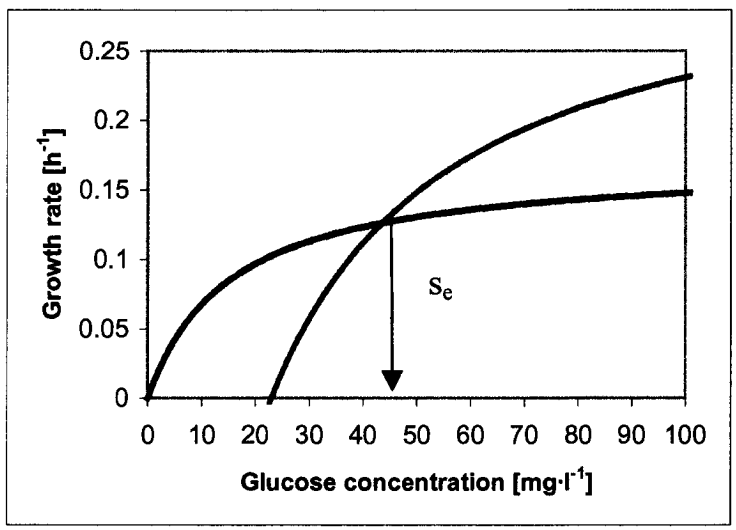

Fig. 5.3 Theoretical glucose concentration (s_e) in a chemostat allowing for co-existence under steady-state conditions assuming extended Monod kinetics (*C. heintzii* (—) and *E. coli* (—)) in a pure and simple competition.

During the high glucose concentration phase *C. heintzii* growths close to its maximum specific growth rate but this is considerably slower than that of *E. coli* (Fig. 5.1c).

The glucose concentration at which the two competitors grow at the same rate can be calculated from equation 4. Graphically, this is the point on intersection of the two growth kinetics curves (Fig. 5.3). Mathematically the solution of equation 3 for s results in equation 4.

$$\mu_{max_{Ch}} * \frac{s}{K_{s_{Ch}} + s} = \mu_{max_{Ec}} * \frac{(s - Smin)}{K_{s_{Ec}} + (s - Smin)} \tag{3}$$

$$s = \frac{\mu_{max_{Ch}} * s_{min} + \mu_{max_{Ec}} * K_{s_{Ch}} - \mu_{max_{Ch}} * K_{s_{Ec}} - \mu_{max_{Ec}} * s_{min} - \sqrt{(\mu_{max_{Ch}} \cdot K_{s_{Ec}} + \mu_{max_{Ec}} * s_{min} - \mu_{max_{Ch}} \cdot K_{s_{Ec}} + \mu_{max_{Ec}} * s_{min})2 - 4 * (\mu_{max_{Ch}} \mu_{max_{Ec}}) \cdot s_{min} \cdot K_{sCh} \cdot \mu_{max_{Ec}}}}{2 * (\mu_{max_{Ch}} - \mu_{max_{Ec}})} \tag{4}$$

$\mu_{max_{Ch}}$: maximum specific growth rate [h^{-1}] with glucose for *C. heintzii*

$\mu_{max_{Ec}}$: maximum specific growth rate [h^{-1}] with glucose for *E. coli*

$K_{s_{Hc}}$: Monod affinity constant [$\mu g \cdot l^{-1}$] for glucose for *C. heintzii*

$K_{s_{Ec}}$: Monod affinity constant [$\mu g \cdot l^{-1}$] for glucose for *E. coli*

s: substrate concentration in continuous culture [$\mu g \cdot l^{-1}$]

s_{min}: extrapolated minimum substrate concentration at D= 0 h^{-1} for *E. coli*

For *E. coli* and *C. heintzii* the glucose concentration for coexistence under steady-state conditions is predicted to occur at 41.25 $\mu g \cdot l^{-1}$ exactly. Under natural conditions there is no chance that this concentration will establish over an extended period of time. It is much more likely that the glucose concentration will fluctuate around this value.

The example shown in Fig. 5.1 and 5.2 demonstrates that coexistence in a fluctuating open system is not only dependent of mutual competition. In addition to the fluctuating growth rate of the competitors it is also the wash-out or death rate that has to remain constant and which will determine the outcome. The basic condition that must be fulfilled for a specific strain to remain in the system is that

over a period of time the constantly washed-out cells can be at least replaced by new cells. Mathematically this is described in equation (5).

$$\int \mu_1 * x_1 * dt \geq \int D * x_1 * dt \quad , \text{ and}$$
$$\int \mu_2 * x_2 * dt \geq \int D * x_2 * dt \qquad (5)$$

$x_{1,2}$: biomass [$mg \cdot l^{-1}$] of organism 1 and 2, respectively
D: dilution rate including death rate [h^{-1}]
$\mu_{1,2}$: specific growth rate [h^{-1}] of organism 1 and 2, respectively

This is visualised in Fig. 5.1d where the concentration biomass of both *C. heintzii* and *E. coli* at the end of the period is the same as at the start. The system is in a fluctuating steady-state. It should be pointed out that two bacterial strains follow quite different strategies to remain in the system. Whereas *C. heintzii* is continuously growing, although at some point fast, than again at a slower pace, *E. coli* experiences an on-off regime with respect to growth (Fig. 5.1c). The latter strategy seems to be more critical from a physiological point of view because the cell has to maintain its activity during the period of scarce glucose supply such that it will be able to switch quickly to growth when glucose becomes available again. Note that a decrease of the residual glucose concentration below s_{min} does not necessarily mean that the cell is not anymore consuming sugar; it simply means that the cell is not growing any more. Hence the ability of the cell to maintain responsiveness during the "starvation" period has to be optimised. One should keep in mind, however, that the nutritional regime in this model system, with glucose as the only carbon/energy source available, are rather harsh. In natural environments the cells are usually not exposed to one single substrate only but to a wide range of carbon sources present at low concentrations (Münster, 1993). It was shown in numerous experiments that under such conditions most microbial cells are able to simultaneously utilise many different substrates even if these individual carbon sources on their own are not sufficient for active growth (Egli, 1995). Hence, the simultaneous utilisation of different carbon sources should allow an opportunist such as *E. coli* to remain at least in an

active physiological state and to be ready to use the next flush of substrate for rapid growth. The set of kinetic parameters in combination with the chosen substrate supply regime in our model system are certainly not the only set of conditions that allows co-existence of *E. coli* and *C. heintzii* and leads to a stable mixed culture. In order to get a feeling of how sensitive the system reacts to changes in the kinetic parameters for growth the border conditions for each of the parameters was determined where the coexistence switched to a dominance(>99%) of either *C. heintzii* or *E. coli* (Table 2).

Parameters	**Dominance of** ***E. coli*** (>99% of the total population)	**Coexistence** (*E. coli* 37% and *C. heintzii* 63% of the total population , kinetic parameters used from Table 1)	**Dominance of** ***C. heintzii*** (>99% of the total population)
$\mu_{max_{Ec}}$	No dominance possible	0.33 h^{-1}	≤0.245 h^{-1}
$K_{s_{Ec}}$	No dominance possible	35 $\mu g \cdot l^{-1}$	≥125 $\mu g \cdot l^{-1}$
$s_{min_{Ec}}$	≤3 $\mu g \cdot l^{-1}$	23.70 $\mu g \cdot l^{-1}$	≥145 $\mu g \cdot l^{-1}$
$\mu_{max_{Ch}}$	≤0.105 h^{-1}	0.17 h^{-1}	≥0.2125 h^{-1}
$K_{s_{Ch}}$	≥52.5 $\mu g \cdot l^{-1}$	15 $\mu g \cdot l^{-1}$	No dominance possible
$s_{min_{Ch}}$	≥200 $\mu g \cdot l^{-1}$	0 $\mu g \cdot l^{-1}$	No dominance possible
D	≥0.1304 h^{-1}	0.075 h^{-1}	≤0.048 h^{-1}
Temperature	≥ 32°C	30°C	≤ 27°C

Table 5.2 Sensitivity analysis of parameters
In order to see how sensitive the population composition reacts to minor changes in the kinetic parameters the range of every parameter assuming the other parameter constant was determined between *E. coli* dominance and *C. heintzii* dominance (in each case 99% of the total population). The same oscillating substrate supply was used as described in Fig. 5.1.

The analysis in Tab. 5.2 indicates that coexistence is possible within a certain range of the kinetic parameters. Whereas for some of the parameters (e.g. $\mu_{max_{Ch}}$) minor changes can already lead to the dominance of one of the strains this range can vary for others (e.g. $s_{min_{Ec}}$) considerably without affecting the possibility of

coexistence. Nevertheless, it seems difficult to predict the outcome of a competition under even more complex conditions than those outlined here.

5.2 Growth rates under environmental fluctuating glucose concentrations

Heterotrophic microbial growth is usually limited in most environmental systems by the scarce availability of carbon/energy sources and temperatures far from the optimum (Egli, 1995; Morita, 1997). In contrast to the continuous supply of glucose in our chemostat experiment a daily variation of carbon compounds easily utilisable for heterotrophic microbes, such as carbohydrates, carboxylic and amino acids, is typical for surface waters. In lakes for example the pattern of free glucose concentration in the photic zone is strongly linked to the photosynthetic activity of algae, which is of course again linked to the daily light income (Münster & Albrecht, 1994). The daily pattern for free glucose concentration in the upper zone of a lake in northern Germany, Lake Plußsee, is shown in Fig. 5.4 (Münster & Albrecht, 1994).

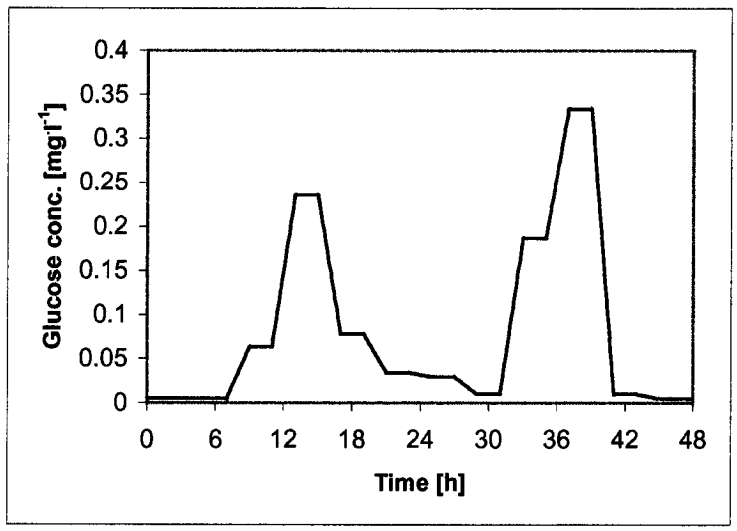

Fig. 5.4 Glucose concentration in the photic zone of Lake Plußsee during a 48 hour period. The pattern is adapted for modelling purposes from data published by Münster (Münster & Albrecht, 1994).

The lake is eutrophic and therefore glucose excreted from algae accumulates transiently to relatively high concentrations (up to 340 $\mu g \cdot l^{-1}$) compared to average free glucose concentrations in open waters (1-10 $\mu g \cdot l^{-1}$)(Wright & Hobbie, 1966; Egli, 1995). The specific growth rate and population dynamics of *C. heintzii* and *E. coli* for this glucose pattern was now simulated for different temperatures based on the kinetic parameters listed in Table 1 and the result is shown in Fig. 5.5 and 5.6. For both strains a common constant decay rate of $0.075h^{-1}$ was used representing predation, autolysis, etc.

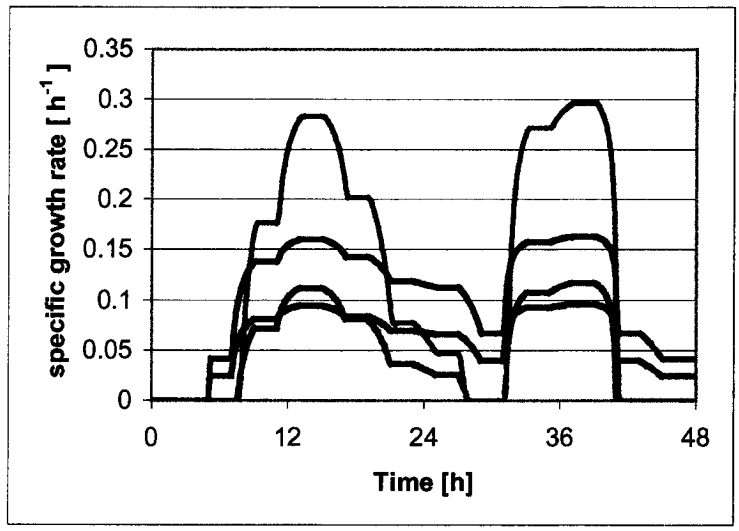

Fig. 5.5 Predicted specific growth rate of *E. coli* and *C. heintzii* in the photic zone of Lake Plußsee during the 48 hour period. At 30°C (*E. coli* (—), *C. heintzii* (—)) and 21°C (*E. coli* (—), *C. heintzii* (—)).

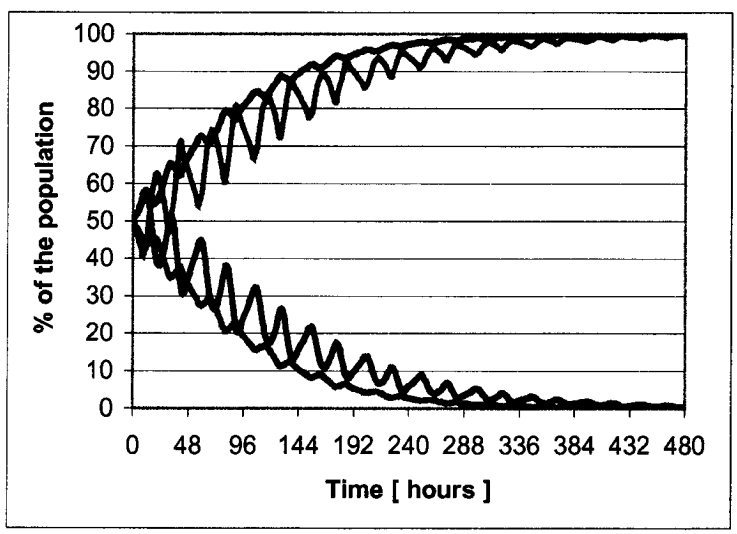

Fig. 5.6 Predicted population dynamics of *E. coli* and *C. heintzii* in the photic zone of Lake Plußsee during the 48 hour period. At 30 °C (*E. coli* (—), *C. heintzii* (—)) and 21 °C (*E. coli* (—), *C. heintzii* (—)).

The results clearly indicate that *E. coli* should have a higher average specific growth rate than *C. heintzii* and, therefore, would outcompete *C. heintzii* under this substrate regime. However, it should be pointed out that the kinetic parameters of *E. coli* and *C. heintzii* were determined at 30°C whereas the

average temperature of this lake, in summer is usually between 17 and 21°C (Overbeck & Chrost, 1994).

5.3 Impact of temperature on the competition

Temperature is one of the most important environmental factors influencing the growth and survival of organisms (Brock et al., 1994). Whereas the influence of temperature on μ_{max} can be investigated easily in batch experiments the dependence of K_s on temperature is difficult to assess and consequently is not well documented. The kinetic parameters listed in Table 1 had been determined at 30°C and for more realistic predictions data for μ_{max} and K_s at environmental temperatures are needed.

For *E. coli* complete set of data is available for growth with glucose not only for μ_{max} and K_s but also for s_{min} (Kovarova et al., 1996a). The data set for these parameters is shown in Fig. 5.7 and 8. Surprising is the independence of K_s on temperature.

For *C. heintzii* the experimental data set was not well documented. Therefore, the dependence of the maximum specific growth rate on temperature for growth on glucose was determined. This bacterium is less thermotolerant, its optimum temperature was at 25°C and growth at 37°C was not detected (Fig. 5.7). However, at lower temperatures the reduction in maximum specific growth rate was not pronounced as in the case of *E. coli*. It was assumed that similar to *E. coli* the K_s of *C. heintzii* was also temperature independent.

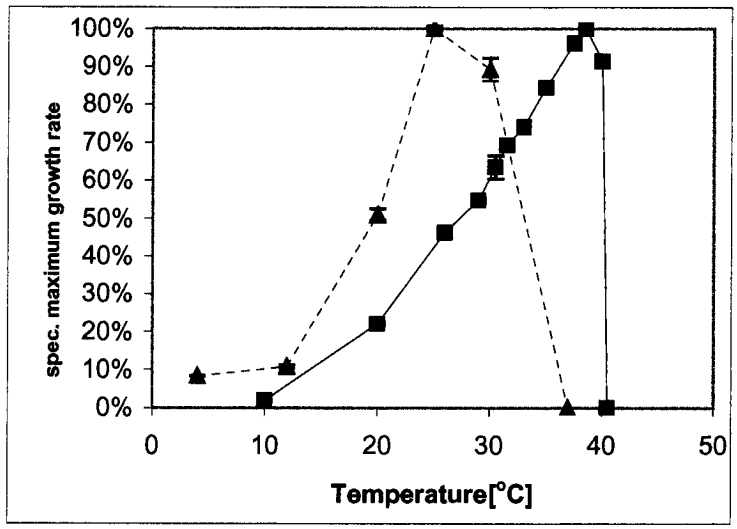

Fig. 5.7 Temperature dependency of the maximum specific growth rate for glucose of *E. coli* (■) from (Kovarova et al., 1996a)and *C. heintzii* (▲).

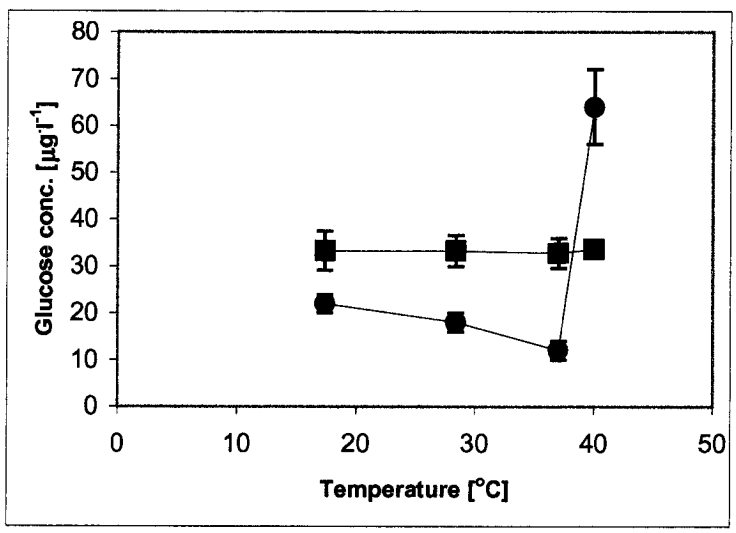

Fig. 5. 8 Temperature dependency of s_{min} (●) and K_s (■) for growth of *E. coli* with glucose from (Kovarova et al., 1996a).

Simulation of a competition between *C. heintzii* and *E. coli* were done for the daily glucose concentration pattern shown in Fig. 5.4. As above a common constant death rate of 0.075 h^{-1} was used to summarise effects due to grazing, cell autolysis and dilution.

Surprisingly, the predicted border between the virtually complete dominance of *E. coli* at temperatures higher than 29°C and the dominance of *C. heintzii* below 29°C was very strict. Hence, according to our simulation results it is not surprising that in tropical zones *E. coli* is a part of the environmental microbial population (Hazen & Toranzos, 1990; Solo-Gabriele et al., 1999) whereas in the northern hemisphere it is generally assumed that *E. coli* can not survive in the environment. Even in hot summer or in shallow surface waters it seems hardly possible that *E. coli* successfully competes for glucose and grows in eutrophic lakes where the water temperature is around 20-25°C during summer time in the northern hemisphere (Eckel & Dobesch, 1986; Sahlberg, 1987).

Conclusions

The main competitive disadvantage of *E. coli* under environmental conditions seems to be its lower substrate affinity, its enhanced maintenance energy requirements (s_{min}) and its slow growth in the temperature range below 29°C. Hence, at elevated temperatures *E. coli* can co-exist and compete with environmental strains and particularly the fluctuating, substrate availability seems to be one of the main factors that allow this enterobacterium to establish itself in ecosystems.

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6. Effect of integration of a GFP reporter gene in *Ralstonia eutropha* on growth kinetics with 2,4-D

Hans Peter Füchslin, Irene Rüegg, Jan Roelof van der Meer, Thomas Egli

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Abstract

Green fluorescent proteins (GFPs) are frequently used as marker and reporter systems to assess the fate and activity of microbial strains with the ability to biodegrade xenobiotic compounds. To evaluate the potential of this tool for tracking pesticide-degrading microorganisms in the environment a GFP reporter system linked to genes coding for the degradation of the pesticide 2,4-D was integrated into the chromosome of a 2,4-D–degrading strain of *Ralstonia eutropha* JMP 134. The effects of the inserted GFP genes on the kinetics of 2,4-D degradation by the GFP clone in batch and chemostat culture were compared with those of the wild type strain.

In batch culture with 2,4-D as the only source of carbon and energy the maximum specific growth rate, μ_{max} (0.228±0.010 h^{-1}) of the GFP clone was virtually the same as that of the wild type (0.225±0.013 h^{-1}). However, compared to the wild type, the 2,4-D steady-state concentration in 2,4-D-limited chemostat cultures of the GFP clone was higher at all dilution rates tested. The kinetics of both the wild type and the GFP strain can be described by an extended Monod model including a term for a minimum substrate concentration for growth (s_{min}).

The consequences of the GFP gene integration into the chromosome for the kinetic behaviour of *R. eutropha* were two-fold. Firstly, it led to a significantly reduced affinity for the substrate 2,4-D from 56.7 $\mu g \cdot l^{-1}$ (K_{swt}) to 167.1 $\mu g \cdot l^{-1}$ (K_{sGFP}), and secondly, to an increase in s_{min} from 16.5 $\mu g \cdot l^{-1}$ (wild type) to 31.6 $\mu g \cdot l^{-1}$ (GFP construct). The reduced competitiveness of the GFP clone at low

substrate concentrations was confirmed in a competition experiment for 2,4-D in continuous culture at $D=0.075h^{-1}$. Reproducibly, the GFP mutant strain was displaced by the wild type strain.

The study clearly demonstrates that fitness of constructs cannot be assessed only by measuring μ_{max} in batch cultures but that a thorough kinetic analysis is needed that also considers slow, carbon-limited growth conditions as they pertain in the environment.

Introduction

Green fluorescent protein (GFP) –carrying strains are now frequently used for tracking specific microbial strains in ecosystems. GFP constructs cells have been employed to study the distribution and dynamics of bacterial populations in soils (Errampalli et al., 1998; Tresse et al., 1998), water systems (Stretton et al., 1998), rhizospheres (Gage et al., 1996; Bloemberg et al., 1997; Tombolini et al., 1997), activated-sludge (Eberl et al., 1997; Olofsson et al., 1998) and biofilms (Möller et al., 1998; Skillman et al., 1998). Furthermore, GFPs can be used to visualise gene expression (Cubitt et al., 1995; Andersen et al., 1998). In such constructs the GFP gene is fused behind the promoter of a particular gene of interest, e. g., a promotor of a biodegradation operon. As soon as environmental conditions allow the induction of the biodegradation pathway the GFP will be expressed, and if sufficiently expressed its fluorescence can be detected in cells by UV light without any addition of substrate.

Being certainly a promising tool to obtain a better insight into microbial processes in the environment the limits of application also have to be evaluated critically. Very little is known about the impact of the integrated marker GFP gene on the metabolism of the constructed strain and whether or not this integration affects the physiological and kinetic behaviour of the construct. In several field studies, a GFP was used as a marker and reporter gene to study the distribution and activity of specific microbial strains in the environment. It was always assumed that GFP strains were not handicaped by the genetic/metabolic burden (Tombolini et al., 1997; Andersen et al., 1998; Errampalli et al., 1999; Dandie et al., 2001; Elväng et al., 2001). Only few and simple experiments are usually carried out to assess whether insertion of the GFP gene has an apparent adverse effect. Control experiments are typically based on whether or not the μ_{max} of the construct is reduced in batch culture compared to the wild type strain (Tombolini et al., 1997; Dandie et al., 2001).

However, for some applications, in particular when using the construct in environmental systems, it is essential to assure that tagging with the GFP does not constitute a discernible metabolic burden to a cell. Nevertheless, as far as the

authors are aware no detailed growth kinetic analysis in batch and continuous culture has ever been reported for a GFP-carrying strain.

All over the world 2,4-dichlorophenoxyacetic acid (2,4-D) still is the most widely used synthetic herbicide to control broadleaf weeds (Bohmont, 1999). The herbicide is biologically degradable and of all described 2,4-D-degrading-bacteria *Ralstonia eutropha* is the best investigated organism with respect to the 2,4-D degradation pathway as well as kinetics of growth and 2,4-D degradation. *R. eutropha* JMP 134 carries a 22 kb DNA region on plasmid pJP4 (Pemberton et al., 1979) on which fifteen genes responsible for degradation of 2,4-D are organised mainly in three clusters (Streber et al., 1987; Perkins et al., 1990; Matrubutham & Harker, 1994; You & Ghosal, 1995; van der Meer, 1997; Leveau et al., 1999; Laemmli et al., 2000). The enzymes for 2,4-D degradation are also responsible for part of the degradation of 3-chlorobenzoate (3CBA), phenoxyacetic acid and cometabolic degradation of trichlorethylene (Don & Pemberton, 1981). The plasmid pJP4 is an 80-kilobase pair broad-host-range, P1 incompatibility group plasmid and further carries resistance determinants to mercury (Don & Pemberton, 1981).

A mutant of *R. eutropha* JMP 134 was constructed containing a fusion between one of the promotors from the 2,4-D degradation pathway, i.e., the *tfdC* promotor the gene for GFP –F64L, S65T. This fusion was integrated into the chromosome. The clone expresses GFP, when the genes encoded for 2,4-D degradation are induced and green fluorescence can be used to measure indirectly the 2,4-D degradation activity of single cells.

In order to assess whether or not expression of GFP is a metabolic burden for the cell resulting in a negative effect on the growth of the construct, the kinetics of 2,4-D degradation of the GFP clone was studied in batch and chemostat culture and was compared to that exhibited by the wild type. Furthermore, direct competition experiments between the wild type strain and GFP clone were carried out in continuous culture to confirm the results obtained.

Materials and methods

Organism and cultivation conditions

Strains

The strain *Ralstonia eutropha* JMP134(pJP4) was used as wild type. *R. eutropha* strain 1265 is a derivative of strain JMP 134 with the tfdc::gfp insertion and kanamycin resistance.

Construction of GFP transconjugants

A 486-bp fragment containing the intergenic region between *tfdT* and *tfdC* (nucleotides 260 and 367, sequence numberings according to GenBank entries U16782 and M35097, respectively) was amplified by the PCR by using primers 990702f (5' CTA GCG GCA GCA TGC GAT CGC 3', *Sph*I site underlined) and 990703r (5' CAT CGA CAA CAT TCT AGA CTC 3', *Xba*I site underlined) and *R. eutropha* JMP134 chromosomal DNA as template. This fragment was cloned into vector pGEM-T-Easy (Promega, Madison, Wis., USA) and its sequence determined to exclude any undesired mutations. The *tfdT-C* intergenic region was retrieved by cutting with *Sph*I and *Xba*I. This fragment was inserted into plasmid pJAMA23, cut with the same two enzymes. Plasmid pJAMA23 (Marco Jaspers, unpublished) contains the *gfp*-mut3 gene encoding the GFP-F64L, S65T mutant protein (Cormack & Falkow, 1996) behind the *rrnB* transcription terminator. This yielded plasmid pCBA 204. Plasmid pCBA204 was digested with *Not*I, the 1.5-kb fragment isolated and ligated with the 7-kb *Not*I vector part of the mini transposon plasmid pCK218 (Kristensen et al., 1995). After transformation into *Escherichia coli* CC118□pir this yielded plasmid pCBA206. The fragment containing the *tfdT-C* intergenic region transcriptionally fused to the *gfp* gene plus the gene for kanamycin resistance was subsequently introduced into *R. eutropha* JMP134 by triparental mating with the help of *E. coli* HB101 (pRK2013) (Ditta et al., 1980) and transposase activity. Transconjugants of *R. eutropha* JMP134 containing the *tfdC::gfp* fusion were selected for kanamycin resistance and subsequently checked for chromosomal integration by Southern hybridisation (not shown). One of the transconjugants, strain 1265, which had the highest GFP expression when grown on 2,4-D, was selected for further studies.

In order to determine the exact site of insertion of the *tfdC::gfp* fragment on the chromosome of *R. eutropha* strain 1265, we used the strategy of rescue of the kanamycin resistance gene. Hereto, total DNA of strain 1265 was partially digested with *Sau*3AI. Fragments in the range between 2.5 and 3.5 kb were isolated and ligated with pUC18, digested with *Bam*HI. After transformation in *E. coli* DH5α, selection was made for kanamycin resistant colonies. The plasmid of one such kanamycin resistant transformants was isolated and found to contain approximately 0.4-kb overlap with the chromosomal insertion site. The insert of this plasmid was completely sequenced, which showed that the *tfdC::gfp* fusion had been inserted in a *pykA* homologous gene for pyruvate kinase.

Medium

Batch medium

The mineral medium contained, per litre: 275 mg NH_4Cl , 75 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot H_2O$, 35 mg KCl, 1.5 mg $FeCl_2$, 60 μg H_3BO_3, 100 μg $MnCl_2 \cdot 4H_2O$, 120 μg $CoCl_2 \cdot 6H_2O$, 70 μg $ZnCl_2$, 25 μg $NiCl_2 \cdot 6H_2O$, 15 μg $CuCl_2 \cdot 2H_2O$, 25 μg $Na_2MoO_4 \cdot 2H_2O$, 5.2 mg $EDTA \cdot Na_4(H_2O)_4$. The mineral medium was supplemented with 2,4-D (99%, Aldrich, Steinheim, Germany) as the only source of carbon and energy. The total 2,4-D concentration was always 500 mg of carbon per litre. After heat sterilisation 100 ml of phosphate buffer ($Na_2HPO_4 \cdot 2H_2O$ / KH_2PO_4 0.56 M with respect to phosphate; pH=7.5) was added by filtration to one litre of medium after cooling down to room temperature using sterile disposable filters (0.22 μm, type GVWP, Millipore, Massachusetts, USA). Also added by filter sterilisation was 0.05 ml of vitamin stock solution per litre of medium. The vitamin stock solution contained per litre: pyrodoxin·HCl, 100 mg; 50 mg of each, thiamine·HCl, riboflavin, nicotinic acid, D-Ca-pantothenic acid, p-amino benzoic acid, lipoic acid, nicotinamide and vitamin B_{12}; biotin 20 mg, and folic acid 20 mg.

Chemostat medium

The mineral medium for continuous cultivation contained per litre: 275 mg NH_4Cl, 1.25 ml H_3PO_4 (85%), 75 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot H_2O$, 35 mg KCl, 1.5 mg $FeCl_2$, 60 μg H_3BO_3, 100 μg $MnCl_2 \cdot 4H_2O$, 120 μg $CoCl_2 \cdot 6H_2O$, 70

μg $ZnCl_2$, 25 μg $NiCl_2 \cdot 6H_2O$, 15 μg $CuCl_2 \cdot 2H_2O$, 25 μg $Na_2MoO_4 \cdot 2H_2O$, 5.2 mg $EDTA \cdot Na_4(H_2O)_4$ and 0.025 ml of silicon antifoam (Fluka, Buchs, Switzerland). The mineral medium was supplemented with 2,4-D as the only source of carbon and energy. The total 2,4-D concentration was always 50 mg of 2,4-D carbon per litre. After autoclaving, 0.25 ml of vitamin stock solution was added per 1 litre of medium by sterile filtration as described above. Chemicals were purchased from either Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany) if not mentioned otherwise.

Cultivation conditions

Continuous cultivation at a dilution rate of 0.075 h^{-1} was performed in a 3.5 litre bioreactor (MBR, Wetzikon, Switzerland) with a working volume 2.8 litre equipped with both pH (7.50 ± 0.05) and temperature control (30±0.1°C). For pH control a mixture of 0.5 M NaOH/KOH was used. The impeller speed was controlled at 750 revolutions per minute and the oxygen saturation was always > 90% air saturation. The bioreactors were regularly checked for wall growth to avoid artefacts as reported by Pirt (Pirt, 1975).

Biomass determination

Biomass measured as cell dry weight was determined by filtration through 0.2 μm pore size polycarbonate membrane filters (Sterico AG, Dietikon, Switzerland). Cells collected on filters were washed once with 50 ml distilled water and filters were dried at 100°C to constant weight. Optical density was determined in a 1 cm cuvette at 546 nm with an Uvikon 860 spectro-photometer (Kontron, Zürich, Switzerland). The correlation factor between dry weight and OD_{546} was determined as 308.1±29.1 [$mg \cdot l^{-1} \cdot OD_{546}^{-1}$].

Analytical procedures

Concentration of 2,4-D in the culture medium was determined by HPLC using a Gynkotek HPLC system consisting of a Gina 50 automated injection module, a M480 G gradient pump, on-line degasser, and a UVD 340S photodiode array detector. Samples collected from the bioreactor were immediately filtered (0.22 μm, type GVWP, Millipore, Massachusetts, USA) and acidified before analysis. 50 μl was injected and the compounds were separated on a Nucleosil column (CC

250/4 Nucleosil 100-5 C18 HD, Macherey Nagel, Düren, Germany) using an eluent consisting of 60% methanol, 40% 50 mM NaH_2PO_4, (pH 4, v/v), at a flow rate of 0.7 ml min^{-1}, and 2,4-D was subsequently detected at a wavelength of 205 nm. The detection limit was 10 $\mu g \cdot l^{-1}$.

Population composition

In competition experiments the population dynamics of the wild-type and the GFP-containing strain was determined by plating on non-selective and kanamycin containing agar plates. The culture from the chemostat was diluted (factor $2*10^5$) in autoclaved phosphate-buffered saline (PBS; 14,4 $g \cdot l^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 3.2 $g \cdot l^{-1}$ $NH_2PO_4 \cdot 2H_2O$, 5.9 $g \cdot l^{-1}$ NaCl, pH 7.5) and five 100 µl aliquots were plated on complex medium agar plates (containing per litre 4 g of tryptic soy agar (Biolife, Milano, Italy) plus 15 g technical agar (Biolife, Milano, Italy)) either with or without 50 $mg \cdot l^{-1}$ of kanamycin (Fluka, Buchs, Switzerland).

The fraction of the GFP strain given in % of the total population was calculated using the following equation (1)

$$P(\%) = \frac{\sum_{z=1}^{5} \text{Number of colonies growing on kanamycin - containing plates}}{\sum_{z=1}^{5} \text{Number of colonies growing on kanamycin - free plates}} * 100\% \qquad (1)$$

Continuous Culture

The dilution rate, corresponding to the specific growth rate of the culture in chemostat is defined as

$$D = \frac{F}{V} \qquad (2)$$

Theoretical wash-out (3) and wash-in (4) curves were calculated by the following equation:

$$s(t) = s(0) \cdot e^{-Dt} \qquad (3)$$

$$s(t) = s_0 \cdot (1-e^{-Dt}) \quad (4)$$

Simulation software

The simulation and parameter estimation was carried out using AQUASIM, a program developed at our institute (Reichert, 1998). AQUASIM allows estimating parameters by fitting calculated values (s_{obs}). This feature was used to estimate kinetical parameters, which resulted in the best fit of simulations and measurements. The program estimated the best fit by minimising χ^2 values ($\chi^2=\sum((s_{obs}-s_{pred})/\sigma)^2$). For the estimation the standard deviation (σ) was set to 10% if not experimentally determined.

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Results

6.1 Growth kinetics with 2,4-D in batch and chemostat culture

6.1.1 Determination of μ_{max} in batch culture

The maximum specific growth rate of both wild type and GFP clone was determined in batch culture with 2,4-D as the only carbon and energy source. *R. eutropha* was adapted to fast growth by transferring the culture from the exponential growth phase at least three times (approximately 12 generations) into new batch medium before the cells were used as an inoculum. Both, the wild type and the GFP-containing strain exhibited perfect exponential growth (Fig. 6.1). The average maximum specific growth rates calculated by linear regression from the logarithmised optical density data were slightly lower for the wild type strain (μ_{max}=0.225 ± 0.013 h^{-1}) than for the GFP strain (μ_{max}=0.228 ± 0.010 h^{-1}). This difference in μ_{max} can not be considered significant because of the overlapping standard deviations.

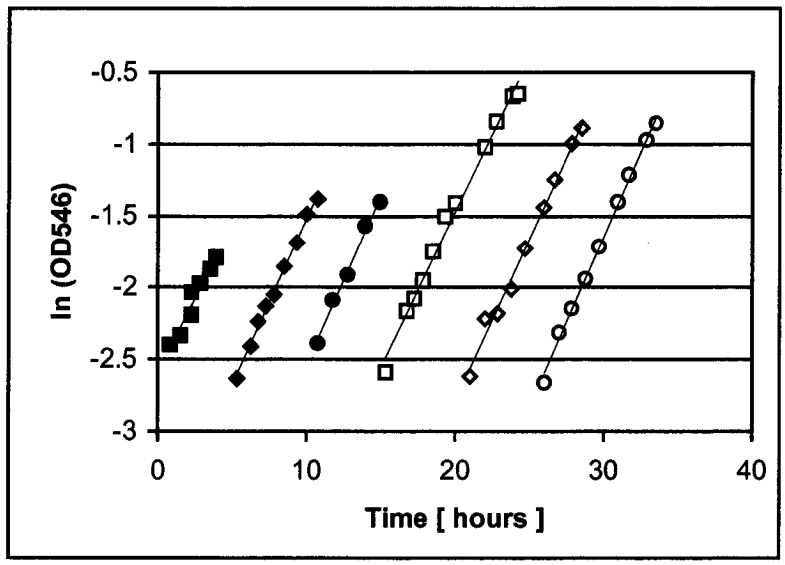

Fig. 6.1 Growth of *Ralstonia eutropha* wild type (■, ◆, ●) and GFP – construct (□, ◊, ○) in batch culture with 2,4-D as the only source of carbon and energy. Growth was followed by measuring optical density at 546 nm. The maximum specific growth rates determined by linear regression were 0.225 ± 0.013 h^{-1} and 0.228 ± 0.010 h^{-1} for the wild type and the GFP construct, respectively.

6.1.2 Growth kinetics in 2,4-D limited continuous culture

Wild type *R. eutropha* and its GFP derivative were grown separately in carbon-limited continuous culture with 2,4-D as the only carbon and energy source and the 2,4-D steady-state concentration was measured as a function of dilution rate. After switching from batch culture to the continuous culture mode the cells were always allowed to adapt first at D= 0.075 h^{-1} at least for one week (approximately 18.2 generations) to the low substrate concentration before sampling was started. At every dilution rate 5 samples distributed over a time period of 5 hours were collected to measure the residual 2,4-D concentration. For a particular dilution rate each steady-state was measured at least twice in independent experiments. After each change in dilution rate the culture was run for 3.5 to 5 volume changes before it was sampled again. Preliminary experiments had shown that this was sufficient time to establish the new steady-state (data not shown). The dilution rate was not changed in an increasing or decreasing order but D was set randomly within the range of 0.025h^{-1} to 0.225 h^{-1}. As soon as slight wall growth was observed the cultures were not used anymore for kinetic experiments. The steady-state 2,4-D concentrations obtained for the wild type and the GFP clone as a function of the dilution rate are shown in Fig. 6.2. The steady-state 2,4-D

concentrations increased with increasing dilution rate as expected for saturation type kinetics (Pirt, 1975). However, at virtually all dilution rates steady–state 2,4-D concentrations in culture of the GFP derivative were distinctly higher than those observed in cultures of the wild type. Only when D was close to D_{crit} 2,4-D steady-state concentrations were similar in both cultures.

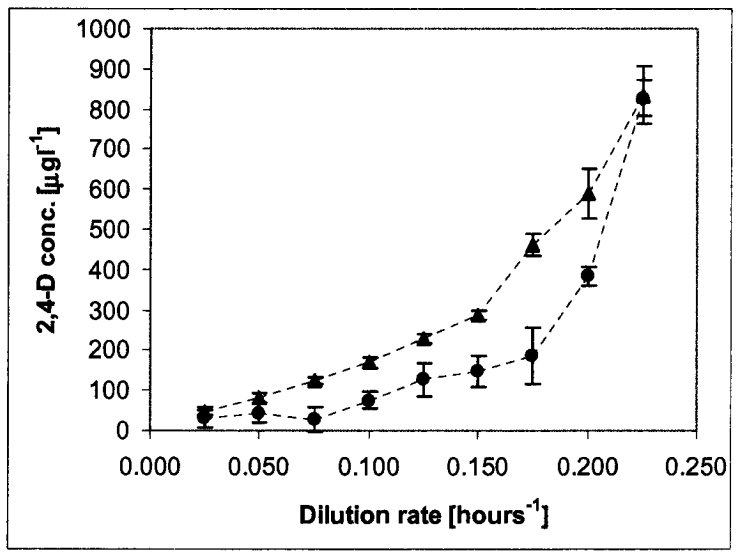

Fig. 6.2 Steady-state concentrations of 2,4-D in carbon – limited continuous cultures of *R. eutropha* wild type (●) and GFP clone (▲), as a function of dilution rate.

6.2 Competition between wild type and GFP strain in continuous culture

To verify the observed kinetic handicap of the GFP derivative a competition experiment was set up with 2,4-D as the only carbon and energy source. Wild type and GFP derivative were first cultivated separately for at least seven days (18.2 generations) in 2,4-D-limited (s_o = 115.02 mg·l^{-1}) continuous culture at a dilution rate of 0.075 h^{-1} in order to allow strains to adapt to low 2,4-D concentration. Then 100 ml of culture was removed from the wild type chemostat and injected into the GFP culture (total working volume 2.8 l) to start the competition on 2,4-D. This should have resulted in a mixture consisting initially of 96% GFP containing plus 4% of wild type cells. Both, total cell number on non-selective complex medium agar plates, and the number of GFP cells on kanamycin–containing TS plates were determined as a function of time to follow

the competition between the two strains. Two competition experiments were carried out, both with the same result (Fig. 6.3). After some 140 hours, corresponding to approximately 15.1 generations, the GFP derivative was displaced by the wild type.

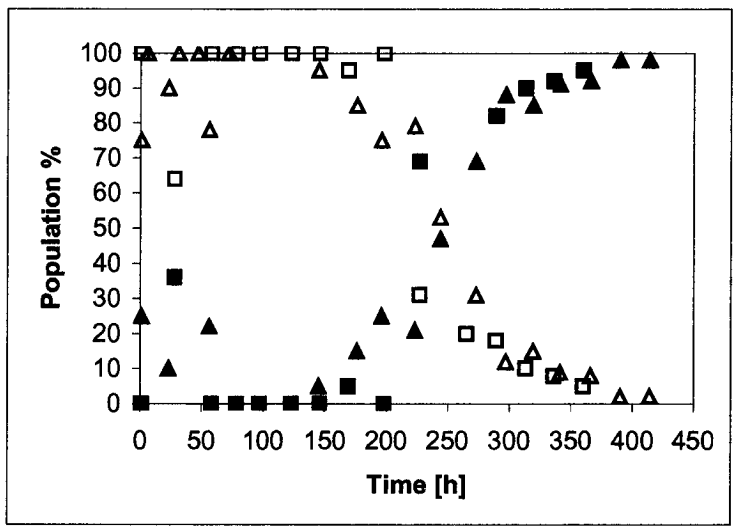

Fig. 6.3 Population dynamics of *R. eutropha* wild type (■, ▲) and GFP clone (□, △) during competition for 2,4-D in two independent 2,4-D-limited continuous cultures at a dilution rate of 0.075 h^{-1}. At time zero the GFP-culture was inoculated with 100 ml of wild type cells precultivated under exactly the same conditions.

6.3 Modelling and simulation of growth and competition

According to the model proposed by Monod (Monod, 1942) two parameters, K_s and μ_{max}, determine the competitive ability of a microbial strain under single-substrate limited growth conditions. Whereas μ_{max} is important for growth at high substrate concentrations in batch culture, K_s is the parameter that mainly affects the ability to compete for substrate at low concentrations and during slow growth (Veldkamp & Jannasch, 1972; Button, 1998; Kovarova & Egli, 1998). The classical Monod equation (equation 5) (Monod, 1942) does not consider effects of maintenance requirements on growth kinetics. Because a number of studies have given evidence for the existence of a minimum substrate concentration for growth, s_{min} (Boethling & Alexander, 1979; Rittmann & Mc Carty, 1980; Schmidt et al., 1985; Alexander, 1994; Tros et al., 1996; Kovarova et al., 1996a), the original Monod equation was modified accordingly (equation 6). In addition, the conversion factor relating substrate utilised to the amount of biomass formed

is needed for modelling the competition (equation 7). The different kinetic parameters for both the wild type and GFP strain during growth with 2,4-D were determined either by parameter estimation (K_s, s_{min}) from the experimental data sets in Fig. 6.2 or from experimental data directly (μ_{max}, $Y_{x/s}$) (Table 6.1).

$$\mu = \mu_{max} * \frac{s}{K_s + s} \tag{5}$$

$$\mu = \mu_{max} * \frac{(s - s_{min})}{K_s + (s - s_{min})} \tag{6}$$

$$Y_{x/s} = \frac{dX}{dS} \tag{7}$$

6.3.1 Modelling growth with 2,4-D in continuous culture

Parameter estimation with the models given in equation 5 and 6 was carried out to obtain the best fit to the experimentally determined 2,4-D steady-state concentrations (Fig. 6.2). The μ_{max} was set within the range of the standard deviation of the experimentally determined value (see above), whereas s_{min} and K_s were allowed to vary and were determined by parameter estimation. The resulting values for K_s and s_{min} are listed in Table 6.1.

For the wild type both models described the experimental data well (Fig. 6.4). Nevertheless, the relative residual plot (Fig. 6.5) indicated that the extended Monod model fitted the data slightly better over the whole range of dilution rates tested and showed a systematical deviation of the original Monod model at low dilution rates.

A more complex pattern was observed for the kinetics of the GFP strain (Fig. 6.6). Obviously, insertion of the GFP gene had a negative effect on the uptake of 2,4-D and residual 2,4-D steady-state concentrations were negatively affected at low rather than at high dilution rates. Possible reasons for this discrepancy to the Monod kinetics will be given in the discussion. Neither the original nor the extended Monod model was able to predict the steady-state 2,4-D concentration reasonably well over the whole range of dilution rates when μ_{max} was set to the

experimentally determined value of 0.238 h^{-1} (Fig. 6.6). Also other models were unable to fit the data over the whole range of growth rates tested, such as the model used by Shehata&Marr (Shehata & Marr, 1971) which assumes operation of two parallel transport systems that both follows Monod kinetics but with different kinetic properties. A reasonable fit was only found with the Monod model extended by s_{min} and multiplied with fitting variable including the impact of changing dilution rate ($\mu=\mu_{max}\cdot(s-s_{min})/(K_s+(s-s_{min}))\cdot D/(D+K_I)$). There is no defined physiological meaning of the fitting variable K_I.

For simplicity and since our competition experiment was performed at a low dilution rate, and μ for neither of the two competing strains exceeded 0.08 h^{-1}, we chose the extended Monod model with the parameters listed in Table 6.1 to model competition of the wild type and the GFP construct (and not the model fitting best over the whole range of dilution rates). The residual plot (Fig. 6.7) visualises the superior fit of the extended Monod model to the experimental data.

		R. eutropha **wild type**	***R. eutropha*** **GFP clone**
Experimentally determined	$Y_{X/2,4\text{-}D}$:	0.226 gg^{-1}±0.022	0.226 gg^{-1}±0.022
	μ_{max}:	0.238 h^{-1}	0.238 h^{-1}
Estimated using original Monod model (eq. 6)	K_s:	71.35 $\mu g \cdot l^{-1}$	211.93 $\mu g \cdot l^{-1}$
Estimated using extended Monod model (eq. 7)	K_s:	56.7 $\mu g \cdot l^{-1}$	167.1 $\mu g \cdot l^{-1}$
	s_{min}:	16.5 $\mu g \cdot l^{-1}$	31.6 $\mu g \cdot l^{-1}$

Table 6.1 Kinetic parameters obtained from parameter estimation by fitting the model to the steady-state 2,4-D concentrations between D=0.025h^{-1} and D=0.175h.

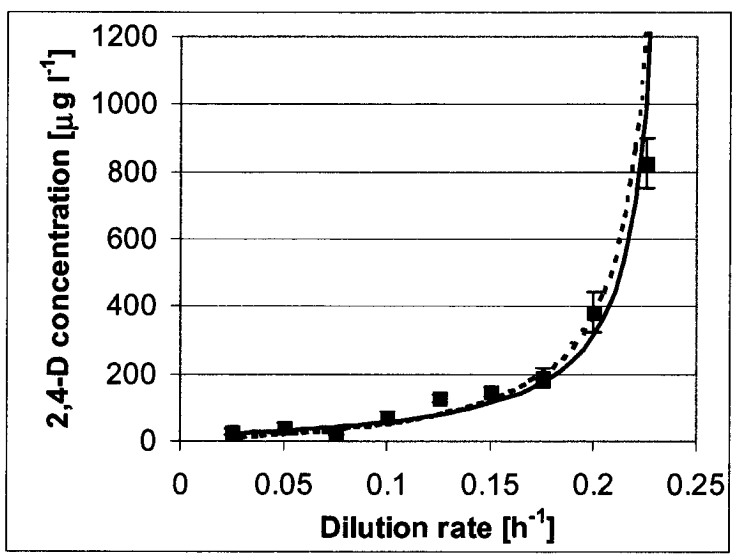

Fig. 6.4 Comparison of model predictions (Monod model (- - -), Monod model extended by s_{min} (——)) and experimentally determined 2,4-D steady-state concentrations (■) for growth of *R. eutropha* (wild type) in 2,4-D limited chemostat culture as a function of dilution rate. For kinetic parameters used see Table 6.1.

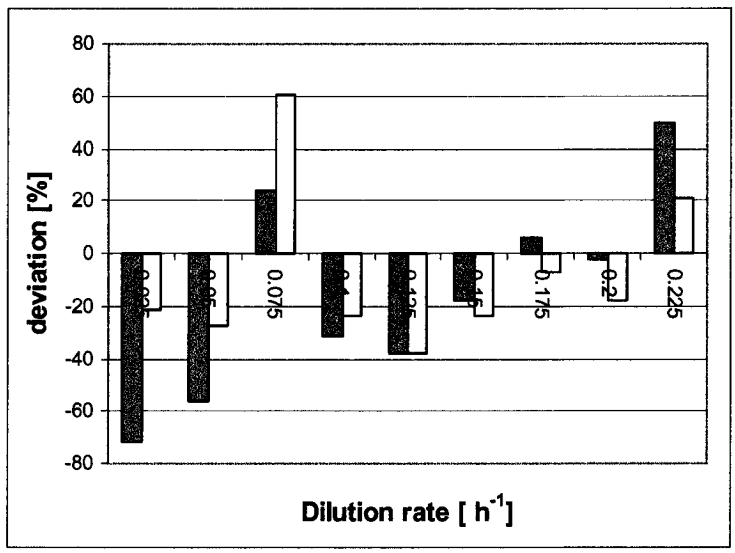

Fig. 6.5 Relative deviations of model predictions (▨ Monod model, □ Monod model extended by s_{min}) from steady-state 2,4-D concentrations for growth of *R. eutropha* (wild type) in 2,4-D limited chemostat culture, as a function of dilution rate. For the simulation by the classical Monod kinetics a weighed deviation (χ^2) of 138.5 was determined compared to the lower χ^2 (77.5) of the extended Monod model.

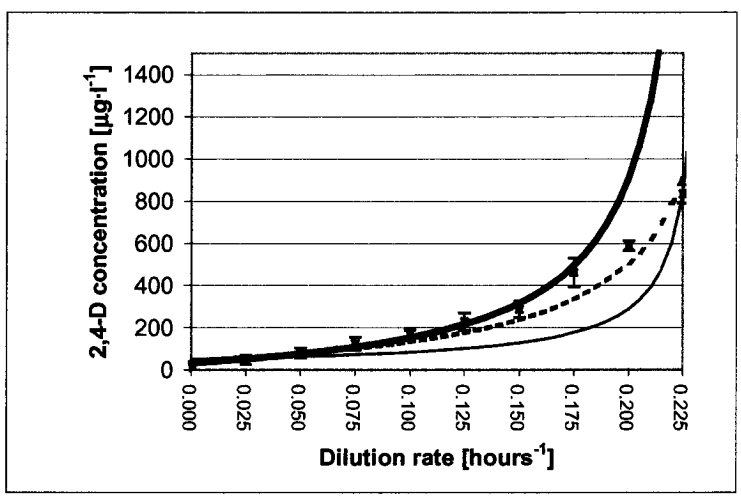

Fig. 6.6 Comparison of model predictions and experimentally determined 2,4-D steady-state concentrations (■) for growth of *R. eutropha* (GFP construct) in 2,4-D limited chemostat culture, as a function of dilution rate. Simulations for three different models are shown: A) Monod kinetics expanded with s_{min} optimised only for low dilution rates (0.025 h^{-1} to 0.175 h^{-1})(—); B) Monod kinetics including a fitting term ($\mu=\mu_{max}\cdot(s-s_{min})/(K_s+(s-s_{min})\cdot D/(D+K_I))$ and kinetic parameter values μ_{max}= 0.238 h^{-1}, K_s=161.3 $\mu g\cdot l^{-1}$, s_{min}=57.96 $\mu g\cdot l^{-1}$, K_I= - 24.99 $\mu g\cdot l^{-1}$ (---); C) Monod kinetics expanded by s_{min} optimised for the steady–state data at the highest dilution rate 0.225 h^{-1} and kinetic parameter values of μ_{max}= 0.238 h^{-1}, K_s=45.33 $\mu g\cdot l^{-1}$, s_{min}=50.47 $\mu g\cdot l^{-1}$ (—).

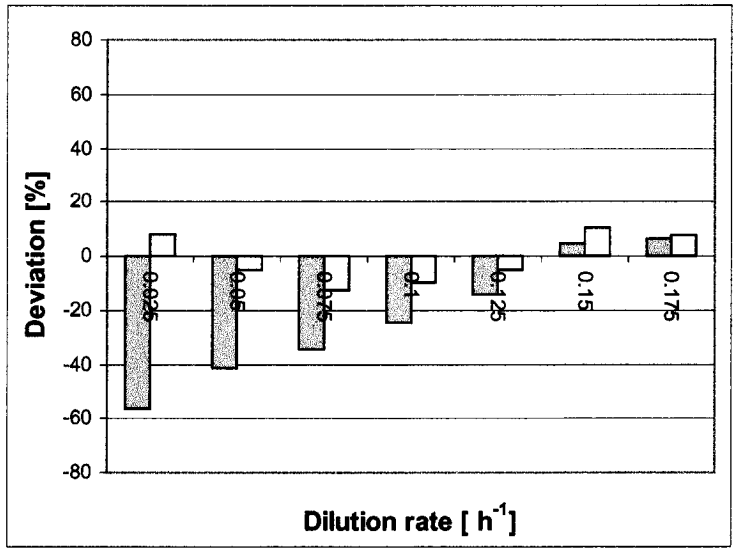

Fig. 6.7 Relative deviations of model predictions (▨ Monod model, □ Monod model extended by s_{min}) from steady-state 2,4-D concentrations for growth of *R. eutropha* (GFP construct) in 2,4-D limited chemostat culture, as a function of dilution rate. The parameter estimation for the kinetic parameters for both models was carried out only for the data at low dilution rates between 0.025 h^{-1} and 0.175 h^{-1}. For the simulation by the classical Monod kinetics a weighed deviation (χ^2) 101.5 was determined which is considerably higher than the χ^2 (10.8) for the extended Monod model.

6.3.2 Simulation of the competition between wild type and GFP clone

The Monod model extended by s_{min} with values for μ_{max}, K_s, s_{min} the kinetic parameters listed in Table 6.1 was used to model the competition experiment between the wild type and the GFP-containing *R. eutropha* strain. A comparison between the experimental data (Fig. 6.3) and the resulting simulation is shown in Fig. 6.8.

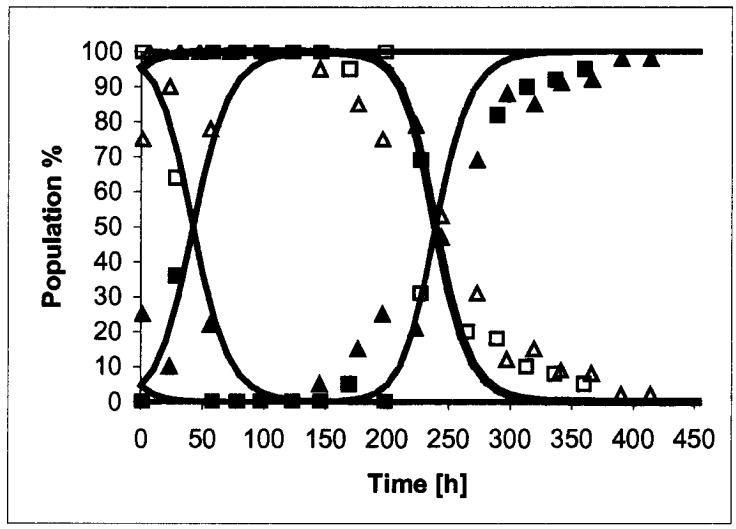

Fig. 6.8 Comparison of model predictions and experimental data for the competition between Ralstonia eutropha wild type (■, ▲) and its GFP clone (, Δ) in 2,4-D-limited chemostat culture at a dilution rate of D= 0.075 h-1. Two different scenarios were chosen to model the lag observed observed. A) Wild type cells were affected by the transfer and started to compete only after an adaptation phase. For this scenario an estimated lag of 100 hours gave the best fit (—). B) Only a part of the wild type cells transferred survived and were viable. For this scenario a viable fraction of 3·10-5 % gave the best fit to experimental data (—). C) Simulation with unaffected inoculum and competition from the beginning (—). For all simulations the extended Monod model and the parameters listed in Table 6.1 were used.

Assuming that the wild type cells transferred into the GFP clone culture were not affected and would start to compete immediately, one would expect the wild type strain to take over the culture after some 100 hours (Fig. 6.8).

The significant delay observed experimentally can be explained in two ways. Either the wild type cells transferred were disturbed and needed a certain recovery time before they were able to compete and to grow. Alternatively, only a minor part of the inoculated cells survived the transfer and were able to grow whereas the rest was not viable and was washed out. With both hypotheses the data can be simulated with good agreement to the experimental results (Fig. 6.8). Only at the end of the competition the complete elimination of the GFP clone from the culture proceeded slower than predicted by the model. This can be

explained by wall growth that started to develop with small portions of the GFP strain sticking to the bioreactor wall, or by its splashing to the bioreactor lid and the subsequent reinoculation from the lid.

In a control experiment it was checked whether growth of the wild type was affected by the excretion of an inhibiting compound by the GFP strain, wild type cells were grown in batch culture on 2,4-D as the only carbon and energy source with and without supernatant collected from a batch culture of the GFP strain. No difference in μ_{max} was observed, suggesting that excretion of an inhibitor compound by the GFP clone is not likely.

6.4 Gene analysis

Since the growth kinetic behaviour of the GFP derivative of *R. eutropha* was different from that of the wild-type strain, it was important to analyse the site of insertion of the *tfdC::gfp* fusion on the chromosome. By using the kanamycin resistance gene rescuing strategy, cloning the fragment into *E. coli* and subsequent DNA sequencing, it was found that the transposon fragment carrying the *tfdC::gfp* fusion had integrated into a pyruvate kinase-like gene (Fig. 6.9). A region of 400 basepairs outside the I-end of the transposon was translated and found to exhibit 50% identical amino acid residues in a 128 residue overlap with the C-terminal end (residues 300-429) of pyruvate kinases from among others, *Mezorhizobium loti* (GenBank entry AP003002), *R. solanacearum* (AL646084) and *Brucella melitensis* (AE009472). This strongly suggests that the transposon inserted into a gene for pyruvate kinase in *R. eutropha* and, possible, interrupted this gene function. However, as far as we know, pyruvate kinase is not involved in 2,4-D degradation. Moreover, many bacterial strains, like *R. solanacearum* and *E. coli* carry two copies for pyruvate kinase.

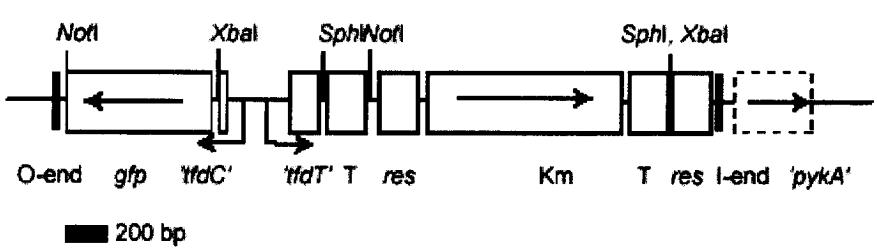

Fig. 6.9 Position of insertion of the *tfdC::gfp* fusion on the chromosome

Discussion

For a GFP-carrying derivative of the 2,4-D-degrading *R. eutropha* both enhanced 2,4-D steady-state concentrations in 2,4-D-limited continuous cultures, and its displacement by the wild type strain during competition for 2,4-D in the chemostat have clearly demonstrated that the GFP derivative was less competitive during slow growth. In contrast, the maximum specific growth rate with 2,4-D was not significantly affected by the insertion of the *tfdC*::*gfp* fusion on the chromosome. The maximum specific growth rate of both the wild and the GFP construct was approximately 0.23 h^{-1} and this rate correspondends well with the values of 0.21-0.215 h^{-1} reported earlier (Greer et al., 1992; Müller & Babel, 1996). Whereas the kinetics of the wild type can be described well with the extended Monod model (eq. 7), the kinetics of the GFP derivative were more difficult to simulate over the whole range of dilution rates tested and a reasonable fit (with simple Monod models) was obtained only at dilution rates smaller than 0.175 h^{-1}. The consequences of the integration of the GFP into the chromosome for the growth kinetic behaviour of *R. eutropha* seem to be twofold. On one hand, it results in a significantly reduced affinity for the substrate 2,4-D from K_{sWT} (56.7 $\mu g \cdot l^{-1}$) to K_{sGFP} (167.1 $\mu g \cdot l^{-1}$), on the other hand it leads to a considerable increase of the minimum substrate concentration from s_{minWT} (16.5 $\mu g \cdot l^{-1}$) to s_{minGFP} (31.6 $\mu g \cdot l^{-1}$). Whereas μ_{max} is consistent with earlier reports both K_s and s_{min} determined here are approximately one order of magnitude lower than those published earlier. For example, the K_s values reported for the 2,4-D degrading *Ralstonia* strain (JMP 134) used by Müller and Babel (Müller & Babel, 1996) was 2830 $\mu g \cdot l^{-1}$ and Greer (Greer et al., 1992) reported a K_s of 2200 $\mu g \cdot l^{-1}$ for growth with 2,4-D. This difference in K_s values may be explained by the longer adaptation time used in our study.

The phenomenon that microorganisms can improve their kinetic parameters during long-term cultivation on a single substrate has already been reported to occur in different experimental systems (Höfle, 1983; Rutgers et al., 1987; Ferenci, 1999; Wick et al., 2001). Similarly, s_{min} determined for growth of the wild type *Ralstonia* strain (JMP 134) in a recycling reactor was 272 $\mu g \cdot l^{-1}$ (Müller & Babel, 1996). This discrepancy might be due to the different cultivation

systems used as already observed by Tros in the case of *Pseudomonas* sp. Strain B13 growing with 3-chlorobenzoate (Tros et al., 1996).
Heterotrophic microbial growth in natural environments is generally limited by the availability of carbon/energy sources (Morita, 1997). Also in natural water bodies or soil contaminated by xenobiotic compounds concentration of available carbon is usually very low (Alexander, 1994; Tros et al., 1996). Hence, our kinetic study suggests that under environmental growth conditions the GFP derivative of *R. eutropha* has a disadvantage compared to the wild type in using low 2,4-D concentrations. The observed handicap must be caused by the additional genetic and/or metabolic burden of the GFP and kanamycin marker, inserted randomly into the genome. Several explanations reasons can be put forward for the reduced competitiveness of the GFP marked strain. One possible explanation could be that the addition of another promotor outtitrates the number of activated TfdR (TfdS) regulatory proteins. Addition of the tfdC::gfp fusion increases the number of TfdR binding sites to four (i.e. tfdA-tfdS, tfdR-tfdDII, tfdT-tfdC and tfdC::gfp) (Leveau et al., 1999). Assuming that in the presence of 2,4-D the concentration of the inducer was the same in both strains a competition for free inducer by the regulatory protein could occur. As a result, the frequency of transcription from the TfdR-regulated promotors might decline, leading to a smaller amount of Tfd gene products. This again might result in a higher residual 2,4-D concentration needed for supporting a particular specific growth rate of the GFP clone. Competition of multiple promoters for the inducer could also explain why at higher dilution rates the difference between the 2,4-D residual concentration of wild type and GFP construct decreased and became virtually equal. At specific growth rates close to μ_{max} the metabolic pools should be virtually saturated and the intracellular concentration of the inducers (Mc Fall et al., 1997) is probably high enough to saturate all promotors. Furthermore, it is also possible that during fast growth several copies of the plasmid pJP4 are present in the cell to support a high growth rate. This implies that the effect of adding an additional promotor on the chromosome is outnumbered by the promotors of tfd operons on plasmid pJ84, leading to a reduced influence on 2,4-D utilisation kinetics. Another explanation for the lower competitiveness of the GFP construct could be that expression of the GFP protein itself led to an increased requirement of maintenance energy reflected in the enhanced s_{min}. Not

only the additionally expressed GFP but also the aminoglycoside phosphotransferase (product of the kanamycin resistance gene) might account for an increased metabolic burden and a reduced competitiveness.
A third reason for the reduced competitive ability of the GFP derivative could stem from the fact that the continuous production and presence of the GFP in the cell might slightly disturb intracellular metabolism and nutrient transport mechanisms (Tombolini et al., 1997). Once formed, the GFP protein appears to be stable and to persist in the cell for long periods of time, probably until the cell lyses (Tombolini et al., 1997). It is only diluted by cell growth and division. Last but not least the site of insertion of the transposon might affect the metabolic behaviour of the construct. For this reason, we analysed the exact insertion site of the *tfdC::gfp* fusion, which turned out to be in/near a putative gene for pyruvate kinase. Although at this point we cannot be completely sure that the *tfdC::gfp* insertion indeed disturbed an intact functional gene for pyruvate kinase on the chromosome of *R. eutropha* JMP134, we believe that even if this was true, it might have only a moderate effect on the kinetic behaviour observed with 2,4-D as growth substrate. First of all, several bacterial strains are known to harbour two copies for pyruvate kinase, such as *R. solanacearum* and *E. coli*, which might also be the case for *R. eutropha*. In fact, the *R. eutropha* GFP derivative still grows on fructose as sole carbon and energy source (unpublished results), suggesting that a functional pyruvate kinase is still present. Secondly, pyruvate kinase is not directly involved in 2,4-D degradation, which proceeds through the formation of 3-oxoadipate, acetyl- and succinyl-CoA and the TCA-cycle (Kaschabek et al., 2002), and, therefore, a defect in pyruvate kinase is unlikely to affect the residual 2,4-D concentration during growth.
Hence, for a number of reasons there is considerable chance that the GFP containing strain might not behave in a similar way as the original wild-type strain. Certainly, the worst mutants, in which the maximum specific growth rate was affected, were eliminated by the initial screening of GFP clones for μ_{max} with 2,4-D in batch culture. As far as the authors are aware, this is the first time that the effect of the integration of a GFP gene into a reporter strain and its expression has been demonstrated clearly under conditions of slow growth. It might be interesting to see whether or not the negative effect of the insertion is restricted to growth with 2,4-D and hence the expression of the GFP. Competition of the wild

type and the GFP clone, e. g. in a fructose-limited continuous culture, might give an answer to this question. Our experiments allowed us to quantify how the chromosomal insertion of the *tfdC*::*gfp* gene fusion affected the kinetics of 2,4-D degradation by *R. eutropha* and our data clearly demonstrate that changes in kinetic properties have to be considered in field experiments with strains containing reporter systems. The work presented suggests that a profound kinetic continuous culture study is needed if one wants to investigate and test the fitness of wild type and genetic engineered strains under environmentally relevant conditions. Our results indicate that the GFP strain investigated here can be used for monitoring the metabolic activity when inoculated into soil containing high 2,4-D concentrations in the range of mg or $g \cdot l^{-1}$, e.g. at the beginning of the bioremediation process. However, the use of this clone for assessing the survival and activity of 2,4-D degrading *R. eutropha* cells in low polluted environments (range of µg or $ng \cdot l^{-1}$) is clearly not feasible. It is very likely that this genetically engineered strain will be out competed by the indigenous 2,4-D degrader populations under carbon-limited oligotrophic conditions.

Definitely, more information is needed in order to construct marker and reporter systems without a significant impact on the host cell. GFPs are a tool of enormeous potential for the investigation of microbial processes in the environment, but as always, their advantages and limitations have to be evaluated critically.

7. Mixed substrate growth of *Ralstonia eutropha* with 2,4-D and fructose

Hans Peter Füchslin, Irene Rüegg, Jan Roelof van der Meer, Thomas Egli

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Abstract

Typical for the growth conditions encountered by microorganisms in most environmental and technical compartments is the presence of mixtures of utilisable carbon sources. Therefore, a key for understanding microbial processes in biological and engineered systems is the knowledge of the principles of mixed substrate utilisation and growth kinetics. As a model system the kinetic behaviour of a *Ralstonia eutropha* JMP 134 cultivated with mixtures of 2,4-D and fructose was elucidated in a carbon-limited continuous culture. The expression dynamics of the catabolic pathway for 2,4-D of *R. eutropha* was investigated by the individual mRNA levels for 5 of totally 15 tfd genes of the 2,4-D pathway during induction of the cells with 2,4-D. Cells were first grown in chemostat under fructose-limiting conditions at a fixed dilution rate of D=0.075 h^{-1}, during which expression of all *tfd* genes was low. Upon exposure to 0.1 mM 2,4-D, there was an instantaneous increase in mRNA levels of all measured *tfd* genes (*tdfB*, *tdfCD*, *tdfE, tdfF*, *tdfK*) followed by the simultaneous utilisation of 2,4-D and fructose. Furthermore, the mixed substrate kinetics of *R. eutropha* growing on different mixtures of fructose and 2,4-D in a carbon-limited continuous culture at a fixed dilution rate of 0.175 h^{-1} was studied. For all mixtures a simultaneous utilisation of 2,4-D / fructose was observed and reduced steady-state concentrations of individual carbon substrates compared to growth with single carbon/energy source was measured. The steady-state concentration of the individual carbon sources was approximately proportional to their fraction in the feed medium. This confirms that the observed principles of mixed sugar kinetics reported earlier can be applied also to mixtures of easily degradable carbon sources and pollutants.

Introduction

In natural and technical environments, microorganisms most probably utilise and grow with many different carbon compounds at the same time (Egli, 1995). However, the existing concepts on microbial growth kinetics are based on the assumption that single substrates are utilised despite the fact that the microorganisms in nature grow with mixtures and under conditions of changing substrate availability. Therefore, it is essential to extend and adapt existing kinetic models especially for the degradation of pollutants in the presence of mixtures of natural substrates.

It is well established that in media containing binary mixtures of carbon substrates in excess the consumption of a particular substrate can be repressed by another easier degradable carbon source. This phenomenon, known as catabolite repression, results in a diauxic growth pattern (Monod, 1942; Magasanik, 1961). In *Enterobacteriacea* glucose is usually the substrate exhibiting the strongest repression effect on the utilisation of other carbon sources (Stumm-Zollinger, 1966; Postma et al., 1993), whereas di- and tricarbonic acids repress the utilisation of sugars in pseudomonads (Ampe et al., 1998).

Less well known is the fact that many micro-organisms do utilise combinations of carbon substrates simultaneously in batch culture even when they are present in excess, particularly when these substrates support only medium or low maximum specific growth rates (for a compilation of data see (Egli, 1995)), and in carbon-limited chemostat culture at low dilution rates the simultaneous utilisation is the rule rather the exception (Egli, 1995; Lendenmann et al., 1996). 2,4-D is the most applied pesticide in the world (Bohmont, 1999). After its usage it is diluted in the soil among a wide range of other natural carbon sources at low concentrations. It is therefore a matter of great practical interest to study the impact of the presence of mixtures of easy degradable alternative substrates on the degradation of 2,4-D.

Ralstonia eutropha is the best investigated organism with respect to 2,4-D degradation pathway as well as its kinetics of growth and 2,4-D degradation. *Ralstonia eutropha* JMP 134 carries a 22 kb DNA region on plasmid pJP4 (Pemberton et al., 1979) on which fifteen genes responsible for degradation of

2,4-D are organized mainly in three clusters as shown in Fig. 7.1A (Streber et al., 1987; Perkins et al., 1990; Matrubutham & Harker, 1994; You & Ghosal, 1995; van der Meer & Leveau, 1997). Characteristic of the 2,4-D (*tfd*) pathway gene organisation is its relatively recent evolutionary formation (Leveau & J.R., 1997) and its mosaic like structure, being composed of DNA fragments of different bacterial origins (Fultthorpe et al., 1995). Among the first identified genes necessary for 2,4-D degradation were *tfdA* (Streber et al., 1987), *tfdB* and *tfdCDEF* (Don & Pemberton, 1985; Perkins et al., 1990). Their products catalyse the sequential conversion of 2,4-D into 3-oxoadipate via 2,4-dichlorophenol (2,4-DCP) and 3,5-dichlorocatechol (3,5-DCC) (Perkins et al., 1990; Fukumori & Hausinger, 1993a; Fukumori & Hausinger, 1993b; Farhana & New, 1997) (Fig. 7.1B). The 22 kb fragment of pJP4 carries an additional set of genes tentatively named $tfdD_{II}C_{II}E_{II}F_{II}$-$B_{II}$ (Fig. 7.1A), based on their similarities to *tfdCDEF* and *tfdB* ranging between 15% and 62% at the amino acid level (Matrubutham & Harker, 1994; Leveau & J.R., 1997). However, their ability to encode a complete pathway for the conversion of 2,4-DCP to 3-oxoadipate has not yet been established. Another newly identified gene on pJP4 is *tfdK*, which encodes a protein facilitating 2,4-D transport (Leveau et al., 1998).

Beside the enzymes for 2,4-D degradation, resistance to mercury, enzymes for the metabolic pathway of 3-chlorobenzoate (3CBA), phenoxyacetic acid and co-metabolic degradation enzymes for trichlorethylene are encoded on the plasmid (Don & Pemberton, 1981). The plasmid pJP4 is an 80-kilobase pair broad-host-range, P1 incompatibility group plasmid (Don & Pemberton, 1981).

Among the many different naturally available carbon substrates, sugars certainly belong to those chemical structural units that are most abundant in nature. Therefore, the 2,4-D degradation pattern exhibited by *R. eutropha* in the presence of the easily degradable carbon source fructose was investigated here. First the response of *R. eutropha* growing on fructose at a low dilution rate to a pulsed addition of a low 2,4-D concentration was studied at mRNA level for genes coding for selected proteins of the 2,4-D degradation pathway. Subsequently, the kinetics of mixed substrate growth of *R. eutropha* was investigated in carbon-limited continuous culture during the simultaneous utilisation of different mixtures of 2,4-D and fructose.

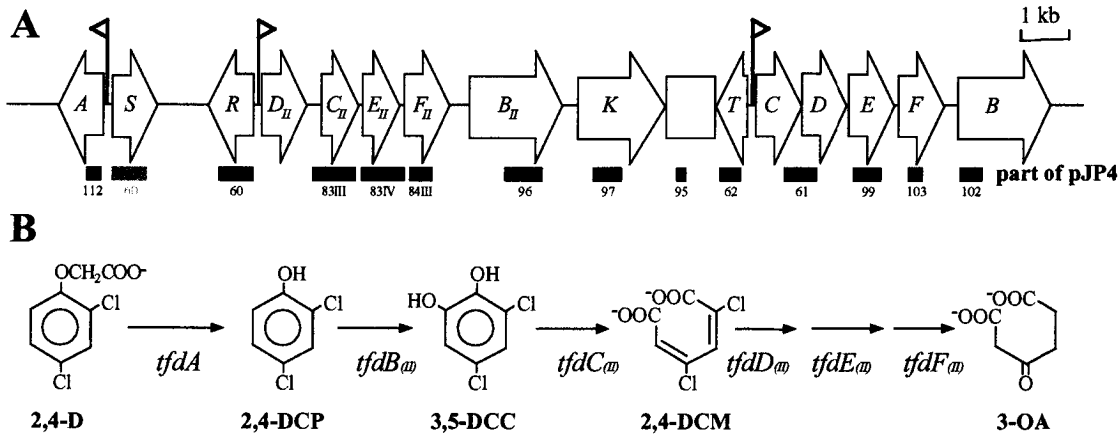

Fig. 7.1 A) Genetic organisation of the *tfd* genes on plasmid pJP4. The orientation and sizes of the genes are indicated by arrows; the solid line is non-coding pJP4 DNA. The rectangle between *tfdK* and *tfdT* represents the IS element ISJP4. Black boxes below the genes indicate locations and sizes of the DNA fragments used for the synthesis of sense and antisense RNA probes; the numbers refer to the corresponding pCBA plasmid constructs (see materials and methodes). Arrows point to the location and direction of known TfdR/S binding sites.

B) 2,4-D catabolic pathway in *R. eutropha* JMP134 (pJP4). Below each conversion step, the gene that encodes the responsible enzyme is indicated (2,4-D, 2,4-dichlorophenoxy-acetate; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; 2,4-DCM, 2,4-dichloromuconate; 3-OA, 3-oxoadipate). 3-OA is further converted to intermediates of the tricarboxylate cycle by chromosomally encoded enzymes.

Materials and methods

Growth conditions

Ralstonia eutropha pJP4 was cultivated continuously at 30°C in a 3.5 l bioreactor (MBR, Wetzikon, Switzerland). This strain is able to use 2,4-D as sole carbon and energy source (Don & Pemberton, 1981). The reactor was operated with a working volume of 2.4 l at dilution rates of 0.05 h^{-1} and 0.175 h^{-1} under carbon-limited conditions. Growth medium for uninduced conditions (medium 1) was based on *Pseudomonas* defined Mineral Medium (Gerhardt et al., 1981) with a pH of 6.8, and with 20 mM fructose. Cells were grown for at least eight volume changes on feed 1 before being exposed to 2,4-D. This was achieved in "block" manner by adding concentrated 2,4-D solution into the reactor to reach a concentration of 0.1 mM instantaneously, and at the same time changing the feed medium to growth medium 2, which was identical to medium 1 but supplemented with 0.1 mM 2,4-D. Throughout the block shift experiment, we monitored changes in the following parameters: mRNA levels of the *tfd* genes (tdfB, tdfCD, tdfE, tdfF, tdfK) and that of the concentrations of biomass, 2,4-D, 2,4-DCP and oxygen consumption. Biomass was followed by measuring optical density (OD) at 600 nm.

Fructose analysis

For the analysis of residual fructose concentrations in continuous cultures of *R. eutropha* the biomass was immediately separated from the culture liquid by rapid filtration (0.22 µm, type GVWP, Millipore, Massachusetts, USA) and acidified as described by (Senn et al., 1994). Analysis included desalting of samples by electrodialysis and subsequent sugar determination by HPLC separation, post-column reaction with alkaline copper(II)bisphenanthroline and amperometric detection. The method for sugar analysis has previously been published in detail (Lendenmann, 1994; Senn et al., 1994).

2,4-D analysis

Concentration of 2,4-D in the culture medium was determined by HPLC using a Gynkotek HPLC system consisting of a Gina 50 automated injection module, a M480 G gradient pump, on-line degasser, and a UVD 340S photodiode array

detector. Samples collected from the bioreactor were immediately filtered (0.22 µm, type GVWP, Millipore, Massachusetts, USA) and acidified before analysis. 50 µl was injected and the compounds were separated on a Nucleosil column (CC 250/4 Nucleosil 100-5 C18 HD, Macherey Nagel, Düren, Germany) using an eluent consisting of 60% methanol, 40% NaH_2PO_4 50 mM (pH 4) (v/v) at a flow rate of 0.7 ml min^{-1}. 2,4-D was subsequently detected at a wavelength of 205 nm with a detection limit of 10 $\mu g \cdot l^{-1}$.

Plasmid constructs for the in vitro synthesis of sense and antisense *tfd* probes

From basically every gene of the *tfd* clusters, short fragments between 250bp and 900 bp were cloned into pGEM-5Zf or pGEM-7Zf (Promega, Madison, USA) and propagated in *Escherichia coli* DH5α (Sambrook et al., 1989). Sources of the *tfd* DNA were cloned fragments from the catabolic plasmid pJP4 of *R. eutropha* JMP134 (Don & Pemberton, 1985; Leveau & Van der Meer, 1996; Leveau & J.R., 1997). All clones were verified by restriction mapping and/or partial DNA sequencing.

In *vitro* synthesis of sense and antisense *tfd* probes

One microgram of each pGEM-derived plasmid (see above) was linearised by restriction enzyme digestion such as to allow insert-specific transcription from either the T7 or the SP6 promotor. *In vitro* transcription reactions were carried out with biotin-16-UTP and T7 or SP6 RNA polymerase as suggested by the manufacturer (Boehringer Mannheim, Germany). After synthesis, the template DNAs were degraded by incubation with RNase-free DNase I (Boehringer Mannheim, Germany), and the RNAs were precipitated. This procedure was performed successfully for all probes, except for *tfd*DII. For unknown reasons, insufficient antisense transcripts were formed from the template DNA of *tfd*DII. Therefore, *tfd*DII was excluded from the analysis. All probes were checked for their specificity in Southern hybridizations with total genomic DNA of *R. eutropha*, digested with different restriction enzymes and with the original plasmids containing the cloned fragments of pJP4 with the *tfd* genes on them. In all cases, the expected banding patterns were observed. Slight cross-hybridisation was found between probes for similar genes (e.g. *tfdC* probe with the *tfdCII* gene); however, these signals could only be observed upon very long exposure

and contributed to less than 1% of the hybridisation signals derived with the true probe target. Antisense probes for *tfdA*, *tfdCD* and *tfdB* were also checked in Northern hybridisation with total RNA isolated from *R. eutropha* during steady-state growth on fructose and 2,4-D (see below). For the *tfdCD* antisense probe, a small cross-hybridisation with the ribosomal RNAs was detected, which may have resulted in over-estimating the background-uninduced level of transcription from the *tfdCD* genes.

RNA extraction

Samples (3-6 ml) were taken directly from the chemostat. Cells spun down in a 30s centrifugation step, and the pellets were immediately resuspended in saline TE (10 mM Tris-HCl, pH 8.0, 1mM EDTA and 100 mM NaCl), buffer II (20 mM sodium acetate, 1 mM EDTA, 0.5% SDS, pH 5.5) and acidic phenol at 60°C as described by Aiba (Aiba et al., 1981). Total RNAs were purified further by phenol-chloroform extraction, precipitation and DNase I digestion (RNase-free). Finally, RNAs were stored with two volumes of ethanol and 0.1 volume of sodium acetate (pH 5.2) at –20°C. Volumetric losses during extraction equalled 40% of the initial sample volume. RNA concentrations were determined spectrophotometrically.

Northern hybridisation of *tfd* mRNAs

Total RNAs (0.1 – 10 µg) from uninduced cells (i.e., during growth with 20 mM fructose) and from induced cells (i.e., 10-20 min after the addition of 2,4-D) were first treated with glyoxylate to denature and were then separated using phosphate-buffered agarose gel electrophereris as described by Ausubel (Ausubel et al., 1995). An RNA size marker (Life Technologies, Rockville, USA) was included on each side of the gel. After electrophoresis, the gels were blotted onto Hybond N-Plus membrane (Amersham, Buckinghamshire, UK) by capillary action. Membranes were treated with UV light in a Stratalinker to fix RNAs irreversibly. Hybridisation was carried out with radioactive-labelled probes at 68°C. As probes, we used DNA inserts of the various pCBA plasmids, recovered by appropriate restriction enzyme digestion and labelled using a randomprimed DNA labelling kit (Boehringer, Mannheim, Germany).

Dot-blot hybridisation of *tfd* mRNAs

Total RNAs were recovered by centrifugation and washing with 70% ethanol, and dissolved in 350 µl of water. Ten-fold (for all time samples) and 100-fold (for samples in peaks) dilutions of the RNAs were prepared. Equal volumes (25µl) of total RNA for each time sample and for each dilution were blotted onto positively charged nylon membranes (Qiagen, Basel, Switzerland) in a dot blot manifold (Gibco Life Technologies, Basel, Switzerland) containing a 96-well 3 mm gasket. Multiple identical blots were prepared directly after each other, as each blot was only used for a single hybridisation. Included on each blot were a series of DNA standards. These standard series were composed of dilutions of the plasmid DNA containing the insert used for producing the complementary antisense RNA and of aliquoted dilutions of total genomic DNA of *R. eutropha* JMP 134 (pJP4). Concentrations of plasmid and total DNA were determined from comparing intensities of serially diluted samples, stained with ethidium bromide and irradiated with UV light, with those of a λ-DNA standard (370 $ng \cdot \mu l^{-1}$; Appligene, Illkirch Grafenstaden, France).

Prehybridisation, hybridisation of the membranes to the antisense biotin-labelled mRNAs and streptavidin-alkaline phosphatase detection of the bound probes was carried out as described previously (Baumann et al., 1996). Membranes were exposed for different time periods to Amersham Hyperfilm MP. Other RNAs isolated from the same chemostats at regular time intervals were dot-blotted separately and used for hybridisation with sense probes. The only exception to this protocol was hybridisation with a probe for the 16S rRNA. This probe consisted of an oligonucleotide (EUB338, 5′-GCTGCCTCCCGTAGGAGT-3′) (Amann et al., 1995) that was labelled with biotin-16-dduUTP using terminal transferase according to the instructions of the supplier (Amersham, Little Chalfont, UK). Hybridisation temperature and conditions for the EUB probe were 2 h at 55°C, after which the streptavidin-alkaline phosphatase detection was carried out.

Quantification of the mRNA levels

Autoradiograms were scanned on a laser densitometer (Molecular Dynamics, Little Chalfont, UK). The density volumes of the dots were quantified with IMAGEQUANT (Molecular Dynamics, Little Chalfont, UK) by applying a grid

on the scanned blot and using the function "local background" for background correction. Quantification data were then processed further with EXCEL (Microsoft, Redmond, USA) and KALEIDOGRAPH PPC (Synergy Software, Reading, USA). mRNA levels were calculated relative to the densities of the DNA standard series and to the amount of total RNA blotted, as outlined below. A non-linear standard curve was obtained by expressing all DNA standards as total amount of *tfd* gene copies per dot versus the observed density volume. For this, we assumed a size of the *R. eutropha* chromosome of 3.5 Mb and a size of 3.5 kb for the pCBA plasmids and, hence, molecular masses of $3.72 \cdot 10^{-6}$ µg and $3.72 \cdot 10^{-9}$µg respectively. As we found previously that pJP4 occurs in monocopy in *R. eutropha* (Leveau & J.R., 1997), this implied that 1 µg of *R. eutropha* JMP134 (pJP4) DNA contained $2.7 \cdot 10^{8}$ *tfd* gene copies (and $5.4 \cdot 10^{8}$ for *tfdR* because of its duplication). Parameters of the non-linear standard curves were derived with KALEIDA-GRAPH using the general hyperbolic equation $y=m1 \cdot m0/(m2+m0)$, where y= the density volume of the dot, m0 stands for the amount of copies per dot, and m1 and m2 are the curve parameters. Parameters that gave the best fit were used to calculate total amounts of *tfd* mRNA copies in each time sample. For peak values (i.e., with the highest density volumes), we used the 100-fold diluted RNAs wherever possible; otherwise, we used the density volumes from the 10-fold diluted RNAs. An average difference between amounts of mRNA copies calculated from undiluted samples versus 10-fold diluted ones was a factor of 9; between 10-fold and 100-fold diluted RNAs a factor of 4. Finally, the amounts of mRNA copies were divided by the total RNA amount blotted (per µg) and by a factor of 10^{8}, assuming that ≈ 1 µg of RNA was isolated from 10^{8} cells (including losses in our sampling procedure). These final values are referred to as relative mRNA levels and can be interpreted as the average mRNA copy number per cell. A specific background hybridisation to DNA contamination in isolated total RNA was low: hybridisation with sense *tfd* probes gave, on average, relative mRNA levels between 0 and 0.03 throughout the experiment (not shown), which were well below the antisense values.

Results

7.1 Pattern of tdf-gene mRNA synthesis upon block-shift exposure of R. *eutropha* to 2,4-D

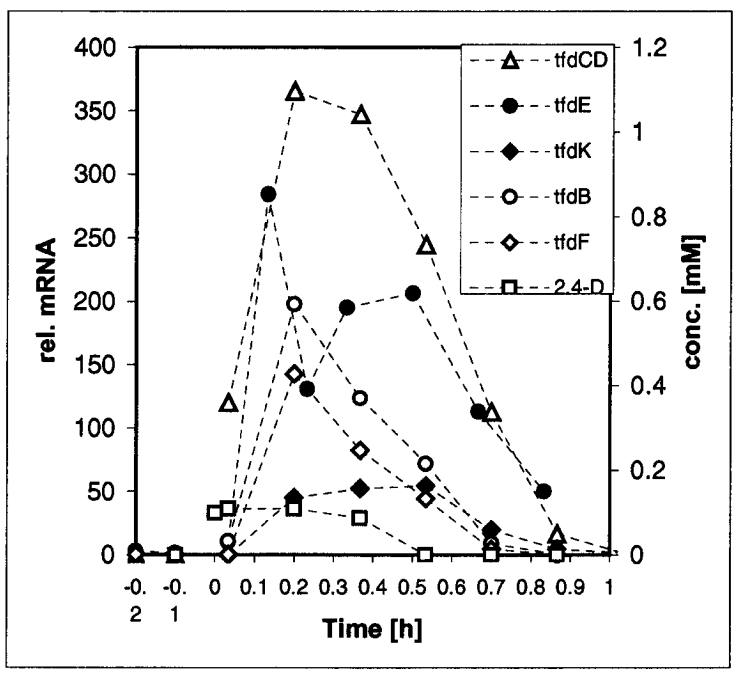

Fig. 7.2 Dynamics of *tfd* gene expression and the changes in 2,4-D and 2,4-DCP concentrations during the first hour after block-shifting *R. eutropha* growing on fructose at D=0.05 h^{-1} to a mixture of fructose plus 0.1 mM 2,4-D.

The short term reaction of *R. eutropha* growing at a dilution rate of 0.05 h^{-1} in carbon-limited continuous culture with fructose (20mM) as the only source of carbon and energy to a block-shift to a medium containing 0.1 mM 2,4-D in addition to fructose (20mM) was studied. Cells were grown for eight volume changes with fructose before they were exposed to the block-shift. The mRNA transcript levels of five mRNA species, namely of the 2,4-D catabolic genes (*tfd* CD, E, K, B and F) and as well as the 2,4-D residual concentration as a function of time after the block-shift (Fig. 7.2) was followed. After the block-shift only one single burst of mRNA synthesis occurred. The transient phase of elevated mRNA synthesis lasted for about 30 minutes and was accompanied by removal of 2,4-D in the reactor to undetectable low levels. Interestingly, no significant

decrease in 2,4-D occurred in the first 20 minutes after the pulsed shift. No 2,4-DCP, a metabolite often excreted by 2,4-D degrading cells and no accumulation of fructose was detected in the supernatant of the chemostat culture during the whole experiment. No significant increase in the optical density (OD) of the culture was detected during is phase (not shown). After the initial accumulation of *tfd* mRNAs their concentrations decreased to steady-state levels, which were generally only slightly above the expression levels of *tfd* mRNAs of uninduced cells (Table 1). The low expression of *tfd* mRNA gene is due to the minor fraction of 2,4-D in the feed and the low growth rate (D= 0.05 h^{-1}). Furthermore these levels were lower for transcripts from genes more distal to the identified TfdR/S binding site i.e., *tfdCD* versus *tfdE* or *tfdF* (Table 1). Hence, after a short lag of 20 minutes during which a burst of *tfd* mRNA's occurred and the synthesis of 2,4-D depending enzymes was initiated, *R. eutropha* utilised fructose and 2,4-D simultaneously.

It was further an attempt made to determine the threshold for utilisation of 2,4-D by exposing a culture growing fructose-limited to continuously increasing concentration of 2,4-D. The inflowing 2,4-D was immediately utilised and no threshold could be determined (Fig. 7.3).

	Uninduced MRNA level[a] [rel. mRNA]	**Peak level during initial phase** [rel. mRNA]	**Steady-state level after block shift during simultaneous utilisation of fructose and 2,4-D** [rel. mRNA]
TfdCD	0.82±0.74	306±197	1.23±0.45
TfdE	0.46±0.32	207±76	1.51±0.25
TfdF	0.93±0.50	143	0.44±0.12[b]
TfdB	1.80±1.72	132±17	1.31±0.30[c]
TfdK	0.87±0.27	65±13	1.95±0.65

Table 7.1 mRNA expression levels of *tfd* gene in *R. eutropha*, before, during and after the block-shift from fructose to a mixture of fructose/2,4-D in continuous culture ($D=0.05h^{-1}$).

(a) Uninduced values are averaged from several experiments (data not shown).
(b) Not significantly different from the uninduced values within the same experiment.
(c) Significantly different from the uninduced values within the same experiment.

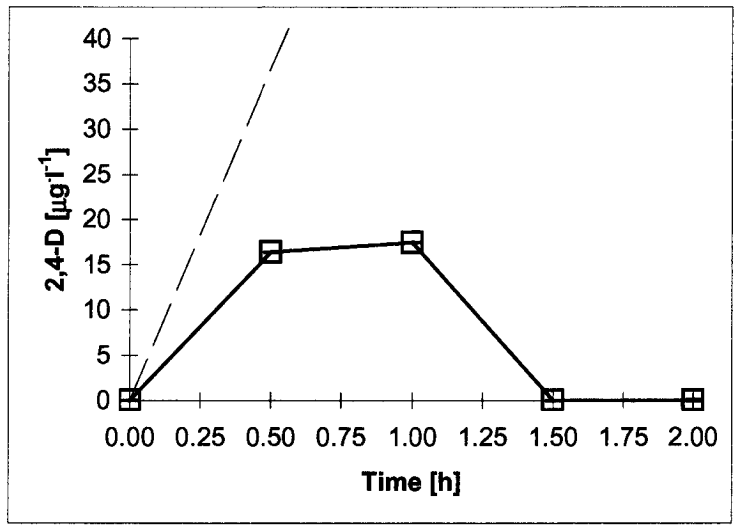

Fig. 7.3 Shift of a culture of *R. eutropha*, growing in a fructose limited chemostat, to a medium containing additional 2,4-D (1 $mg \cdot l^{-1}$).
$S_{0(fructose)}$ was kept constant at 50 mg fructose $C \cdot l^{-1}$. The dilution rate was 0.075 h^{-1} throughout the experiment. 2,4-D concentration (□) was monitored. Theoretical Wash-in curve (---).

7.2 Mixed substrate kinetics

R. eutropha GFP was cultivated in carbon-limited continuous culture at a constant dilution rate of 0.175 h^{-1} first with 2,4-D only and subsequently with mixtures of 2,4-D and fructose where the proportion of 2,4-D in the mixture was increased stepwise.

Throughout the experiment the total concentration of carbon in the feed medium remained constant at 50 mg total carbon·l^{-1}. After changing the feed mixture the culture was always allowed to grow for five volume changes before analysis was started. The residual concentration of 2,4-D and fructose in the culture was measured as a function of the mixture composition supplied in the feed (Fig. 7.4). During growth with all mixtures *R. eutropha* utilised fructose and 2,4-D simultaneously. The residual concentration of fructose and 2,4-D was reduced during simultaneous utilisation compared to growth with single substrates. The residual steady-state concentrations of fructose and 2,4-D reflected approximately their contribution to the total organic carbon in the feed medium. This mixed substrate pattern is similar to previous observations (Egli et al., 1986; Bally, 1994; Lendenmann et al., 1996; Kovarova & Egli, 1998). Based on the steady state biomass concentration measured during mixed substrate growth it can be concluded that biomass was synthesised from both substrates with the normal yields determined during growth with either fructose or 2,4-D as the only carbon/energy source.

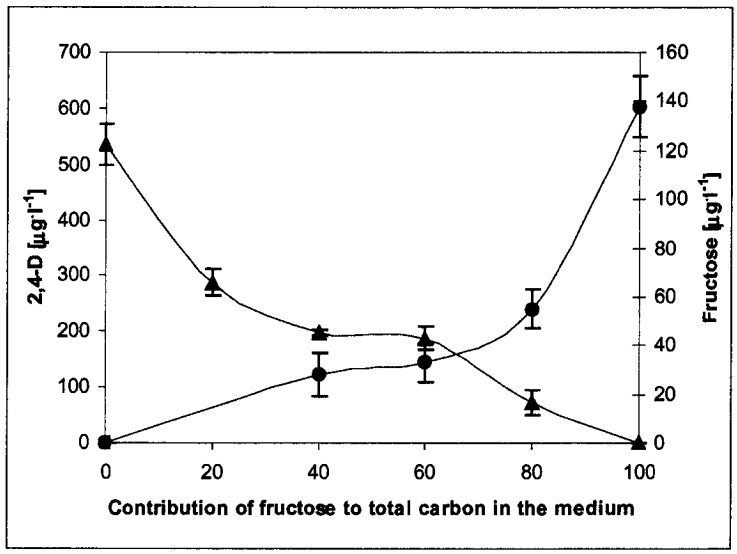

Fig. 7.4 Steady-state concentration of 2,4-D (▲) and fructose (●) during growth of *R. eutropha* in carbon-limited continuous culture at a constant dilution rate of D=0.175 h^{-1} with mixtures of fructose plus 2,4-D in the feed. The mixture composition is given as the contribution of fructose carbon (in %) to the total carbon from both fructose plus 2,4-D.

7.3 Modelling mixed substrate (2,4-D / fructose) utilisation

The experimental data obtained for the mixed substrate utilisation of *R. eutropha* were modelled in two computer simulations. The first simulation was based on extended Monod kinetics (equation 1-3) using the kinetic parameters of Table 1. This simulation deviated significantly from the experimental data (Fig. 7.5). A second simulation was close using Lendenmanns kinetics for mixed substrate utilisation (equation 4) (Lendenmann et al., 1996) It showed a reasonable similarity except for growth with the pure substrates, where the experimentally monitored 2,4-D concentration was significantly higher than those predicted by the model. Interestingly it seems that only addition of minor part of an alternatively carbon source leads to a significantly decrease in the residual concentration of the main carbon source.

$$\mu_{fruc} = \mu_{maxfruc^*} \frac{s_{fruc}}{K_{sfruc} + s_{fruc}} \qquad (1)$$

$$\mu_{2,4-D} = \mu_{max} * \frac{(s_{2,4\text{-}D} - s_{min2,4\text{-}D})}{K_{s2,4\text{-}D} + (s_{2,4\text{-}D} - s_{min2,4\text{-}D})} \quad (2)$$

$$\mu_{total} = \mu_{fruc} + \mu_{2,4-D} \quad (3)$$

$$s = s_{100\%} * \frac{S_{o,i}}{\sum S_{o,i}} \quad (4)$$

	2,4-D	Fructose
μ_{max}:	0.238 h^{-1} (a)	0.433 h^{-1} (b)
K_s (estimated):	138.2 $\mu g \cdot l^{-1}$ (a)	203.5 $\mu g \cdot l^{-1}$ (c)
s_{min} (estimated):	38.9 $\mu g \cdot l^{-1}$ (a)	

Table 7.2 Kinetic parameters used for the simulation of the mixed substrate utilisation of 2,4-D and fructose by *R. eutropha*.
(a) Values from chapter 4
(b) From literature (Müller & Babel, 1996)
(c) Calculated from experimentally determined steady-state fructose concentration: $K_{sFruc} = \mu_{maxFruc} * \frac{(\mu_{max\,Fruc} - D) * s}{D}$

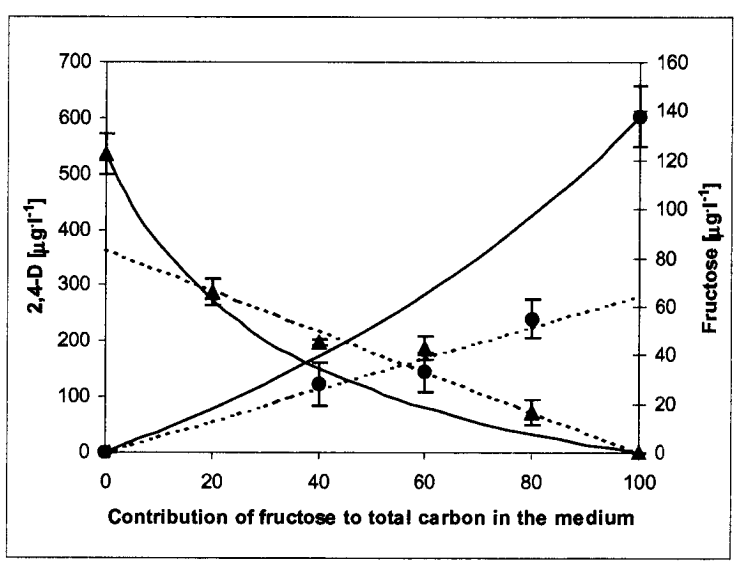

Fig. 7.5 Comparison of model predictions (Monod's model (—) and Lendenmann's model (---)) and experimentally determined 2,4-D (▲) and fructose (●) steady-state concentrations.

Discussion

The first signs of the cellular response of *R. eutropha* growing with fructose when exposed to 2,4-D as new substrate are changes in mRNA transcript levels, rather than enzyme activities. Virtually immediately after addition of 2,4-D the expression of *tfd* mRNA increased to high peak levels that were 33.4 to 325-fold higher than established levels needed for steady-state expression of the enzymes. It can be clearly stated that fructose and 2,4-D was utilised simultaneously after a short induction time (20 minutes), which gives strong evidence that heterotrophic microorganism will utilise mixtures of carbon substrates under carbon-limited conditions in the chemostat (Egli et al., 1986; Bally, 1994; Lendenmann et al., 1996; Kovarova & Egli, 1998). The short induction time can be explained that the presence of second easily degradable carbon/energy source (in our case fructose) was able to accelerate the induction and expression of 2,4-D-degrading enzymes, a phenomena already reported for *Chelatobacter heintzii* growing on NTA/glucose mixtures (Bally & Egli, 1996). It would be of further interest to see if in a shift experiment from fructose medium to a medium containing only 2,4-D the induction time would increase significantly. Furthermore, the concentration of 2,4-D and fructose lowered approximately to its proportion in the feed during mixed substrate utilisation compared to growth at the same dilution rate with the single substrates only.

R. eutropha is adapted to an oligotrophic environment, where it is exposed to a wide range of different carbon sources at low concentration. Therefore, it seems reasonable that it will utilise simultaneously different substrates in order to reach the highest possible growth rate or at least to guarantee the maintenance of the cell at low substrate concentrations.

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8. General Conclusion

What has this study contributed to the knowledge about mixed substrate utilisation and competition?

The work reported considerably extends the present knowledge concerning growth kinetics of microbial cultures with mixtures of carbon substrates. First of all it confirms earlier observations made for the growth of *E. coli* with mixtures of up to six different sugars (Lendenmann et al., 1996), or with glucose plus 3-phenylpropionic acid (Kovarova et al., 1996b). Also here it was found that the simultaneous utilisation of mixtures of carbon substrates by *E. coli* (glucose and lactose, by both the wild type and the *lac* constitutive population), and by the pesticide-degrading *Ralstonia eutropha* (with fructose and 2,4-D), allows the cells to grow at reduced steady-state concentrations compared to growth with single substrates same growth rate. Hence, the experimental data obtained reenforce the suggestion that mixed substrate growth enables cells to grow fast at low concentrations of growth-limiting carbon substrates and that this kinetic principle can be applied generally to growth of microbial cultures, whatever strain and whether growing with natural or xenobiotic compounds. The results obtained also clearly confirmed the competitive advantage cells gain by simultaneously growing with mixtures of carbon substrates.
Convincing evidence has been given here for the existence and the importance of threshold concentrations for both induction of catabolic enzymes, and for the utilisation and growth. The existence of minimum substrate concentrations, s_{min}, needed for maintenance, or for distinct metabolic processes such as the induction of enzyme systems or growth, has been postulated already some 40 years ago. However, so far hard experimental evidence has been very scarce (Alexander, 1994; Egli, 1995; Tros et al., 1996). Here, a minimum concentration of approximately 2 $mg \cdot l^{-1}$ was documented for the induction of lactose-utilising enzymes in wild type cells of *E. coli*. Interesting is the fact that this threshold concentration for induction was not setting the threshold for utilisation as well. One the cells were induced and lactose-utilising enzymes were expressed the cells were able to grow with this sugar also at concentrations far below the

threshold for induction. A similar observation was previously made for growth of this enterobacterium with 3-phenylpropionic acid (Kovarova et al., 2002). Obviously, such a threshold for induction is not only observed for "exotic" substrates for *E. coli*, such as aromatic compounds, but also for carbon sources of natural origin. In addition to a threshold for induction of lactose and 3ppa catabolism, *E. coli* also exhibits a minimum substrate concentration for growth with glucose. The competition experiments with *C. heintzii* described in this thesis suggest that it was not so much the poorer affinity of *E. coli* for glucose, which was the reason for the inability of the enterobacterium to compete during growth with glucose at low dilution rates in continuous culture. The main disadvantage of this bacterium seems to be the existence of an enhanced s_{min} of approximately 20 $\mu g \cdot l^{-1}$ required for growth. In contrast to *E. coli*, no detectable s_{min} was observed for growth under identical conditions for the "gleaner" type of organism *C. heintzii*. One can speculate that these differences in maintenance requirement are characteristic for environmentally successful bacterial strains and copiotrophic species, such as *E. coli*.

When employing the experimentally obtained kinetic parameters for *E. coli* and *C. heintzii* for modelling growth and competition with glucose in the environment good evidence was obtained for a possible co-existence of the two strains conditions as they pertain in surface waters, namely during fluctuating glucose supply. Furthermore, based on the kinetic parameters growth and successful competition of *E. coli* with environmental strains was predicted for growth at temperatures higher than 29°C. This is consistent with the reported survival and growth of *E. coli* in tropical zones where surface water temperatures are elevated compared to temperate climate zones.

For the first time it was clearly demonstrated in this work that integration and expression of foreign genes might have a kinetic disadvantage during growth at low, environmental substrate concentrations. The consequences of the integration of the GFP gene into the *R. eutropha* chromosome for the kinetic performance during growth with 2,4-D was two-fold. Firstly, it led to a significantly reduced affinity for the substrate, and secondly, it resulted in an increase in s_{min}. In contrast, the maximum specific growth rate with 2,4-D as the only carbon source

was the same for the wild type and the GFP construct. Usually it is only μ_{max} which is checked for in order to check whether or not such mutant strains are affected in their growth by the integration of a foreign gene. Our study with *R. eutropha* demonstrates that this is not sufficient. If reporter strains are to be used to monitor the survival and competition of pollutant degrading microorganisms in environmental systems at least a competition experiment with a wild type strain in continuous culture is required. So far we do not know the exact reasons for the kinetic handicap of our reporter strain. However, such knowledge is urgently needed as a background for the construction of reporter strains with kinetic properties matching those of the wild type.

Outlook

Numerous kinetic data is available today for microbial growth. However, our knowledge is almost entirely confined to the kinetic properties of organisms exhibiting when they grow unrestrictedly with short doubling times in batch culture, or at medium to high dilution rates in continuous culture ($D \geq 0.2\ h^{-1}$). The lack of kinetic data during slow growth is understandable if one considers the problems commonly encountered in such investigations. They range from waiting until steady-states have established, wall growth developing during extended cultivation times, analytical problems in analysing to low residual substrate concentrations to proper sampling techniques that ensure the detection of the actual concentration of the growth –limiting substrate, and many more. However, under natural conditions slow growth or even starvation is rather the rule than the exception. In the environment bacteria grow at rates in the range of 0.00.. to 0.1 at the best (Egli, 1995; Morita, 1997) and even in the intestine enterobacteria grow with an average doubling time of 24 to 12 hours (Lee, 1985).
This points out that researchers should design their laboratory experiments such that they simulate as closely as possible the growth conditions microbial cell encounter in ecosystems. Besides many other ecological aspects, this means to carry out experiments at low growth rates.
As a result of my investigations I consider the following question to be of special interest:

First of all, it appears to me of primary interest to clarify whether or not our observation can be generalised that the main kinetic disadvantage of *E. coli* was the existence of an enhanced minimum substrate concentration required for growth, in contrast to *C. heintzii*. This would be an important difference between environmentally successful bacteria and copiotropic genospecies such as enterobacteria. I feel this could a crucial point for the better understanding of the survival of enterobacteria in natural environments such as surface waters. Secondly, the understanding of mixed substrate kinetics in complex system is only in its beginning. Many more studies are needed to understand better the basic mechanisms of mixed substrate utilisation, of growth and competition in engineered and natural environments. It was shown here that not only general substrate kinetic models are necessary but that also the genetic evolution of cultures during long-term exposure to substrate mixtures has to be taken into account and that here many discoveries can be made and surprises are waiting for us. Finally, our experiments strongly suggest that more knowledge is needed on the construction of strains that carry marker and reporter genes, such that they are not affected on the host cell in their kinetic properties during slow growth in the environment. Certainly, GFPs and other reporter systems are a tool of enormous potential for the investigation of microbial processes in the environment, but their advantages and limitations have to be evaluated critically.

Curriculum Vitae

29 March 1970	Born in Brugg (AG)
1976-1982	Primary school in Brugg
1982-1986	Secondary school in Brugg
1986-1990	Alte Kantonsschule in Aarau (Gymnasium) Maturatyp C
1990-1996	Study of Environmental Science, Swiss Federal Institute of Technology (ETHZ): Diploma of Natural Sciences
1993	Promoted to sub-lieutenant of the military medical service
1996	Diploma work at Swiss Federal Institut for Environmental Science and Technology (EAWAG)
1996-2001	PhD student at the Swiss Federal Institute for Environmental Science and Technology (EAWAG) and Swiss Federal Institute of Technology (ETH)
1997-1999	Teaching Assistant at the Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
1997-2001	Teaching Assistant at the University of Basel, Biozentrum, Basel, Switzerland
1999	Promoted to captain of nuclear and chemical weapon protection service
Since February 2002	Detection of microorganisme in the drinking water at Spiez laboratory, a special division of the defence procurement agence in the Swiss Federal Department of Defence

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Appendix 1: Single substrate kinetic models (Extended Monod Models)

Reference	Comment	Growth model $\mu=f(s)$	Residual concentration $s=f(\mu)$	Parameter
(Monod, 1942)	Original Monod model	$\mu = \mu_{max} * \frac{s}{K_s + s}$	$s = K_s \frac{\mu}{\mu_{max} - \mu}$	μ_{max}: maximum specific growth rate K_s: affinity constant
(Pirt, 1975)	Original Monod model extended by smin	$\mu = \mu_{max} * \frac{(s - s_{min})}{K_s + (s - s_{min})}$	$s = K_s \frac{\mu}{\mu_{max} - \mu} + s_{min}$	μ_{max}: maximum specific growth rate K_s: affinity constant s_{min}: predicted substrate concentration at D= 0 h-1
(Powell, 1967)	Model of Herbert	$\mu = (\mu_{max} + m) * \frac{s}{K_s + s} - m$	$s = K_s \frac{(\mu + m)}{\mu_{max} - \mu}$	μ_{max}: maximum specific growth rate K_s: affinity constant m: maintenance rate
(Powell, 1967)	Model Moser	$\mu = \mu_{max} * \frac{s^r}{K_s^r + s^r}$	$s = \left(K_s \frac{\mu}{\mu_{max} - \mu} \right)^{\frac{1}{r}}$	μ_{max}: maximum specific growth rate K_s: affinity constant r: arbitrary exponent
(Contois, 1959)	Biomass dependence of kinetics	$\mu = \mu_{max} * \frac{s}{K_s * x_{rel} + s}$	$s = K_s \frac{\mu * x_{rel}}{\mu_{max} - \mu}$	μ_{max}: maximum specific growth rate K_s: affinity constant
(Powell, 1967)	Diffusion limits	$\mu = \mu_{max} * \frac{K_s + L + s}{2 * L} \left[1 - \frac{4 * L * s}{(K_s + L + s)^2} \right]$	$s = L \frac{\mu}{\mu_{max}} + K_s \frac{\mu}{\mu_{max} - \mu}$	μ_{max}: maximum specific growth rate K_s: affinity constant L: diffusion constant
(Dabes et al., 1973)			$s = \mu * A + K_s \frac{\mu}{\mu_{max} - \mu}$	μ_{max}: maximum specific growth rate K_s: affinity constant A: constant for slow reaction

(Shehata & Marr, 1971)	Two transport system kinetics	$\mu = \mu_1 * \frac{s}{K_1 + s} + \mu_2 * \frac{s}{K_2 + s}, \mu_{max} = \mu_1 + \mu_2$		μ_1 : μ_{max} of high affinity system μ_2 : μ_{max} of low affinity system K1 : K_s of high affinity system K2: K_s of low affinity system
		$\mu = \mu_1 * \frac{s}{K_s + s} + (\mu_{max} - \mu_1) * \frac{s}{K_s + s}$		
(Alexander, 1994)	Model of Haldane	$\mu = \mu_{max} * \frac{s}{K_s + s + (\frac{s^2}{K_I})}$		μ_{max} : maximum specific growth rate K_s : affinity constant K_I: inhibition constant
(Baloo & Ramkrishna, 1991)	Cybernetic model	$\mu = \mu_{max} * \frac{e}{e^{max}} * \frac{s}{Ks + s}$		μ_{max} : maximum specific growth rate K_s : affinity constant e: specific level of enzyme expression (cybernetically regulated) e_{max}: maximum enzyme expression

Table 1 Modified Monod equations

Appendix 2: Alternative models single substrate kinetic models

Reference	Comment	Growth model $\mu=f(s)$	Residual concentration $s=f(\mu)$	Parameter
(Blackman, 1905)		$\mu = a * s \text{ for } s \le \frac{\mu_{max}}{a}$ $\mu = \mu_{max}$	$s = \frac{\mu}{a} \text{ for } \mu \le \mu_{max}$ $s > \frac{\mu}{a} \text{ for } \mu = \mu_{max}$	μ_{max}: maximum specific growth rate a: increase of specific growth rate per substrate
(Powell, 1967)	Tessier	$\mu = \mu_{max}\left(1 - e^{\frac{-s}{T}}\right)$	$s = -T * \ln\left(1 - \frac{\mu}{\mu_{max}}\right)$	μ_{max}: maximum specific growth rate T : Temperature
(Westerhoff et al., 1982)		$\mu = a + b * \ln(s)$	$s = e^{\frac{\mu - a}{b}}$	
(Koch, 1999) (Odum, 1971)	Logistic equation $\mu=f(x)$	$\mu = \frac{r * (K - x)}{K}$		x: biomass r: maximal growth rate K: carrying capacity

Table 2 Alternative kinetic models for single substrate utilisation

Appendix 3: Models for mixed substrate utilisation

Reference	Comment	Growth model μ=f(s1,s2)	Parameter
(Bell, 1980)	Addition of Monod terms	$\mu = \sum \mu_{max.i} * \frac{s_1}{K_i + s_i}$	$\mu_{max,i}$: μ_{max} for growth with substrate i K_i: Monod saturation constants of substrate i
(Gondo et al., 1978)		$\mu = \frac{\mu_{max,1} * s_1}{K_1 + s_1 + \frac{K_1}{I_2} * s_2} + \frac{s_2}{s_1 + s_2} * \frac{\mu_{max,2} * s_2}{K_2 + s_2 + \frac{K_2}{I_1} * s_1}$	$\mu_{max,1}$: $\mu_{max,2}$: μ_{max} for growth with substrate 1 or 2 K_i, K_2: Monod saturation constants of substrate 1 or 2 I_1: inhibition constant of substrate 1 on substrate 2 I_2: inhibition constant of substrate 2 on substrate 1
(Slaff & Humphrey, 1986)		$\mu = \frac{\mu_{max,1} * s_1}{K_1 + s_1} + \frac{\mu_{max,2} * s_2}{(K_2 + s_2) * (1 + B * s_1^2)}$	$\mu_{max,1}$: $\mu_{max,2}$: μmax for growth with substrate 1 or 2 K_i, K_2: Monod saturation constants of substrate 1 or 2 B: inhibition constant of substrate 1 on substrate 2
(Bley & Babel, 1992)		$\mu = \mu_{max} * \left(\frac{p * s_1}{K_1 + s_1} + \frac{(1-p) * s_2}{(K_2 + s_2)} \right)$	μ_{max}: maximum specific growth rate K_i, K_2: Monod saturation constants of substrate 1 or 2 p: proportion of substrate 1 in the mixture
(Baloo & Ramkrishna, 1991)	Cybernetic Model	$\mu = \sum_i \mu_{max\,i} * \frac{e_i}{e_i^{max}} * \frac{s_i}{K_{s,i} + s_i} * v_i$	μ_{max}: maximal growth rate K_s: affinity constant e: specific level of enzyme expression (cybernetic regulated) e_i^{max}: maximum enzyme expression vi: cybernetic control variable for growth activity
(Yoon et al., 1977)	For 2 substrates	$\mu = \frac{\mu_{max,1} * s_1}{K_1 + s_1 + a_{1,2} * s_2} + \frac{\mu_{max,2} * s_2}{K_2 + s_2 + a_{2,1} * s_1}$	$\mu_{max,I}$: μ_{max} for growth with substrate I K_i, K_2: Monod saturation constants of substrate 1 or 2 $a_{1,2}$: inhibition constant of substrate 2 on substrate 1 $a_{2,1}$: inhibition constant of substrate 1 on substrate 2
	Generalised form (> 2 Substrates)	$\mu = \sum_{i=1}^{n} \frac{\mu_{max,1} * s_1}{K_i + \sum_{j=1}^{n} a_{i,j} * s_j}$	$\mu_{max,1}$, $\mu_{max,2}$: for growth with substrate 1 or 2 K_i, K_2: Monod saturation constants of substrate 1 or 2 $a_{i,j}$: inhibition constant of substrate j on substrate i

(Lendenmann, 1994)		$\mu = \frac{\mu_{max} * \sum a_i * s_i}{\mu_{max} + \sum a_i * s_i}$, $s_i = s_{100\%,i} * \frac{S_{o,i}}{\sum S_{o,i}}$	μ_{max}: maximum specific growth rate a_i: specific affinity for growth with substrate i $s_{100\%,i}$: steady state concentration of substrate i during single substrate growth $S_{o,i}$: Inlet concentration of substrate i

Table 3 Kinetic models for mixed substrate utilisation